Helminth glycans and their interaction with the immune system

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Helminth glycans and their interaction with the immune system

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Abbreviations

APC	antigen presenting cell
Asn	Aspargine
BCIP	X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
CAA	Anodic antigen
CCA	Cathodic antigen
CD	Cluster of differentiation
cDNA	copy Deoxyribonucleic acid
C. elegans	*Caenorhabditis elegans*
Cer	Ceramide
CLR	C-type lectin receptor
CMP	Cytidine monophosphate
CRD	Carbohydrate recognition domain
DC	Dendritic cell
DCIR	DC immunoreceptor
DC-SIGN	DC-specific ICAM3-grabbing nonintegrin
DDA	dimethyl dioctadecyl ammoniumbromide
Dex	dexamethason
DF-LDN-DF	(Fucα1-2Fucα1-3)GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc
Dol	Dolichol
dsRNA	double-stranded Ribonucleic acid
D. viviparus	*Dictyocaulus viviparus*
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
ES	Excretory secretory protein
ESI-MS	Electrospray ionization mass spectrometry
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal calf serum
F. hepatica	*Fasciola hepatica*
F-LDN	Fucα1-3GalNAcβ1-4GlcNAc
F-LDN-F	Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc
Fuc	Fucose
Gal	Galactose
Gal-3	Galectin-3
GalNAc	Galactosamine
GDP	guanosine diphosphate
GL	Glycolipid

Glc	Glucose
GlcA	Glucuronic acid
GlcNAc	Glucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GS1B4	Griffonia simplicifolia 1-B4
H. contortus	*Haemonchus contortus*
HEK 293	Human Embryonic Kidney 293 cells
HF	hydrogen fluoride
HPLC	High performance liquid chromatography
ICAM3	intercellular adhesion molecule-3
IFN-γ	Interferon-γ
Ig	immunoglobulin
IL-1	Interleukin-1
IRAK	IL-1 RI-associated protein kinases
LAMP	Lysosome associated membrane protein
LBP	Lipopolysaccharide-binding protein
LDN	LacdiNAc GalNAcβ1-4GlcNAc
LDN-DF	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc
LDNF	GalNAcβ1-4(Fucα1-3)GlcNAc
Le^X	$Lewis^X$ Galβ1-4(Fucα1-3)GlcNAc
Le^Y	$Lewis^Y$ Fucα1-2Galβ1-4(Fucα1-3)GlcNAc
L. major	Leishmania major
LN	LacNAc: Galβ1-4GlcNAc
LNFPIII	Lacto-N-fucopentaose III
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
L-SIGN	Liver/lymph node-specific ICAM3-grabbing nonintegrin
mAb	monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption ionization/time of flight
Man	Mannose
Mda-5	melanoma differentiation-associated gene 5
MGL	macrophage galactose-type C-type lectin
MHC	major histocompatibility complex
MR	Mannose receptor
mRNA	messenger Ribonucleic acid
MyD88	Myeloid differentiation factor 88
NBT	4-Nitro blue tetrazolium chloride
NeuAc	N-acetylneurominic acid
NF-κB	Nuclear factor-kappa B
Nod	Nucleatide-binding oligomerization domain

PAA	polyacrylamide
PAMP	Pathogen-associated molecular pattern
PCR	polymerase chain reaction
pNP	p-Nitrophenyl-N-acetyl
PRR	Pathogen/pattern recognition receptor
PS	phosphatidylserine
Pseudo Le^Y	Fucα1-3Galβ1-4(Fucα1-3)GlcNAc
RIG-I	Retinoic acid inducible gene I (RIG-I)
S. mansoni	*Schistosoma mansoni*
S. haematobium	*Schistosoma haematobium*
S. intercalatum	*Schistosoma intercalactum*
S. japonicum	*Schistosoma japonicum*
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEA	Soluble egg antigen
Ser	Serine
Sia	Sialicacid
ssRNA	Single-stranded Ribonucleic acid
T. canis	*Toxocara canis*
TIR	Toll/IL1 receptor domain
TGF-β	Transforming growth factor-β
Th	T-helper
Thr	Threonine
TLR	Toll-like receptor
TNFα	Tumour-necrosis factor α
TRIF	TIR-domain-containing adapter-inducing interferon-β
T. spiralis	*Trichinella spiralis*
UDP	uridine diphosphate
Vit D3	1α,25-Dihydroxyvitamin D_3 or vitamin D3
Xyl	Xylose

CHAPTER 1

General introduction





Helminths infections – also known as metazoan or worm like parasite infections - are a major cause of suffering and economical loss in mammalians worldwide [1, 2]. In the spectrum of pathogens helminths are an exceptional group because they are large multicellular organisms, designated from free-living metazoan ancestors. Although grouped together, helminths consist of two different phyla that are evolutionary very distantly related [3]: the nematodes or roundworms and the platyhelminthes which are subdivided into two classes named cestodes (tapeworms) and trematodes (flukes). These classifications are made on the basis of internal and external morphology of eggs, larval and adult stages [4]. Individuals in the different groups have evolved to occupy a wide range of niches in the mammalian host, ranging from the intestinal lumen to intravascular and even intracellular sites, using a diverse variety of infection strategies. Helminths are exogenous to the host and enter the host by ingestion or by penetration of the anatomical barriers by themselves or via a vector (e.g. insect bites). Although there is a high diversity between the different helminths, the mammalian host response to them is remarkably consistent [5, 6].

Unlike many bacterial and viral infections, helminth infections are often chronic, lasting for months or even years. Helminth infections are generally not fatal, but the rate of morbidity is mostly high and chronic infections lead to anaemia and malnourishment. In developed countries these infections are better controlled due to the primary health care programs, public sanitation and control of insect vector populations. In developing nations, on the other hand, the problem of helminth infections is widespread and the rate of re-infection after treatment is high [7].

The relationship between helminths and the host immune system is the result of a long co-evolution. The parasite has the best possibility to survive if it is able to trick the host into developing an ineffective immune response, so it can mature in the host and start reproduction. Of importance in this process is that the helminth does not cause such damage that the host is killed or seriously harmed. The host on the other hand needs to generate an effective immune response to expel the parasite and minimize the harmful effects of both the parasite and the tissue damage caused by the immune response.

An enormous amount of effort has already been put in the identification of parasite structures that could be used as effective vaccine components or diagnostic tools in helminth infections. In particular, the development of vaccines is becoming increasingly important due to the increasing resistance of helminths against antihelminthic drugs [8-10]. The development of a vaccine could in the end result in reduction of the morbidity and economical losses associated with helminth infections. In the past, research has been mainly focussed on the use of recombinant protein antigens as vaccine components, without much success [11-13]. In the last decade, several research groups have relayed their focus on glycan antigens of helminths. Glycans, linked to proteins and lipids, are abundantly expressed on the outer surface of helminths and on their excretory/secretory products, which makes them accessible to the host immune system. In recent years, many studies have shown that helminth glycan antigens play an important role in the induction of both cellular and humoral immune responses [14-17]. However, much is left to be

learned about the molecular mechanisms by which the helminth glycans act. Helminth glycans have been associated both with immune activation, as well as with immune suppression, which makes it very interesting to investigate the influence of helminth glycoconjugates on the immune system and to characterize the glycans which play a role in inducing these responses. More insight in the immunomodulatory capacity of helminth glycans may open the way to the development of glycan-based vaccines or immunotherapeutical treatment of infectious disease or chronic inflammatory diseases.

In this dissertation, different aspects of the role of helminth glycans in their interaction with the immune system are highlighted. The two helminth species that were studied, namely the gastrointestinal nematode *Haemonchus contortus* and the trematode *Schistosoma mansoni,* are shortly introduced in the next paragraphs.

Haemonchus contortus

H. contortus is also known as 'red stomach worm', 'wire worm', or 'barber's pole worm'. This blood sucking gastro-intestinal nematode belongs to the family of Trichostrongylidae. The lifecycle is initiated when the adult female worm starts producing eggs (5000-10000 eggs daily), which are passed out in the faeces of the animals. The eggs continue to develop in the faeces in moist conditions and hatch into the first larval stage (L1). The second larval stage (L2) molts out of the L1 by emerging from the cuticle, a protective layer around the larvae. The L1 and L2 are free-living pre-parasitic stages that live from feeding on the bacteria in the faecal material. The L2 molts into the third stage larvae (L3) but keeps the cuticle of the L2. This cuticle prevents the L3 from feeding, as a result it has to rely on the nutrients stored by the L2 larvae. The L3 larvae crawl up the wet grass to be eaten by goat/sheep. The L3 larvae do not survive long when conditions are dry and the temperatures are high. Infectious larvae are swallowed by the host and pass through the first three stomachs towards the abomasum. During infection, the L3 stage molts into the L4 stage or immature adult form. The L4 stage develops a lancet with which it pierces the abomasums lining and starts feeding on blood of the host. It shields from the host by covering itself in a blood clot. After a few days, the worm matures, emerges from the clot and attaches to the mucosa of the abomasum and starts producing eggs thereby completing the lifecycle [18] (Figure 1).

Distribution and pathology

The nematode *H. contortus* is a worm that parasitizes sheep and goats in areas with a warm climate. The economical loss is worldwide due to the loss of milk, meat and wool products and frequent death of young animals because of blood loss [2].

The most obvious symptoms of infection are progressive weight loss and anaemia. Animals may become anaemic during the course of infection if left untreated, with pale gums and inside of eyelids and even death as result. Iron and albumine levels may drop which leads to "bottle-jaw", a condition that is the consequence of oedema of the lower jaw and the neck region [19, 20].

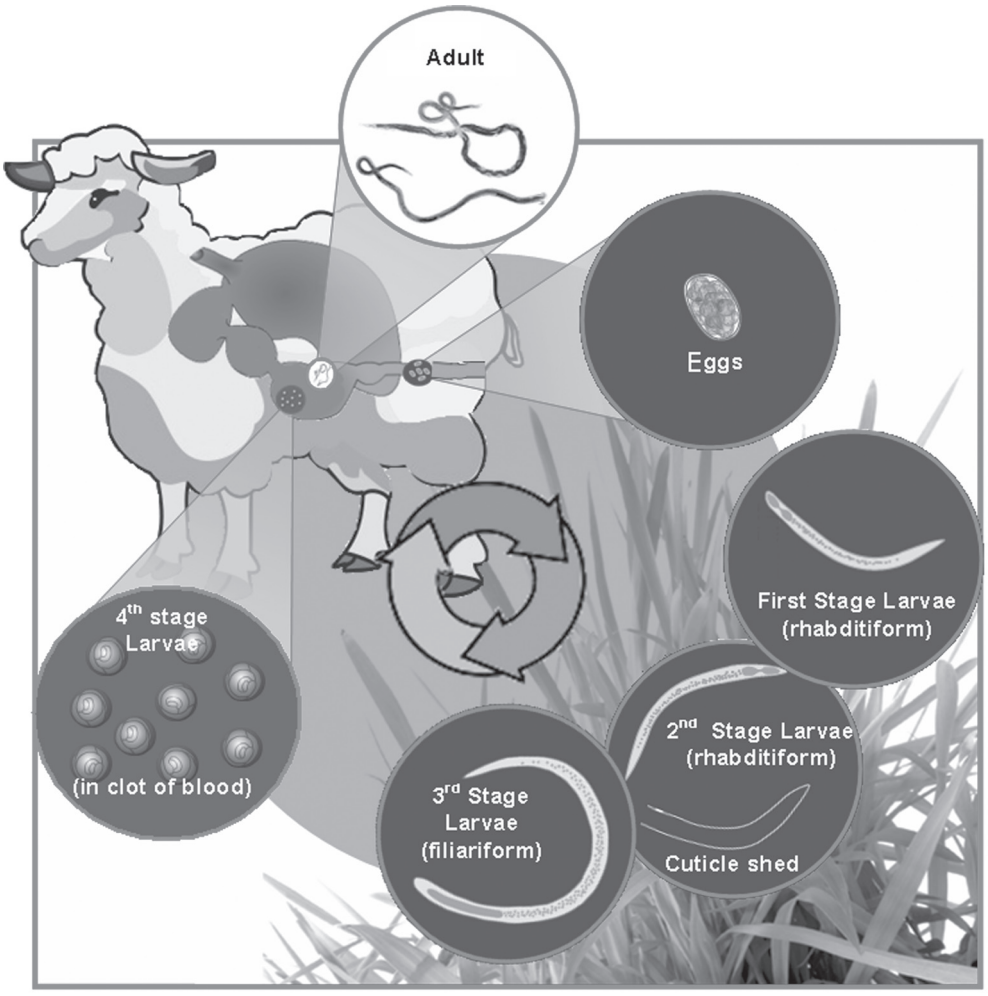

Figure 1: lifecycle *Haemonchus contortus.*

The lifecycle of *H. contortus* is initiated when the adult female worm starts producing eggs, which are passed out in the faeces of the animals. The eggs continue to develop in the faeces and hatch into the first larval stage (L1). The second larval stage (L2) molts out of the L1 and the L2 molts into the third stage larvae (L3). The L3 larvae crawl up the wet grass to be eaten by goat/sheep. Infectious larvae are swallowed by the host and pass through the first three stomachs towards the abomasum. During infection, the L3 stage molts into the L4 stage or immature adult form. The L4 stage shields itself from the host by covering itself in a blood clot. After a few days, the worm matures, emerges from the clot and starts producing eggs thereby completing the lifecycle.

In the case of highly acute haemonchosis, sheep may suddenly die as a result of hemorrhagic anaemia caused by severe blood loss form the gut [21].

Over the years several breeds of sheep were found to be genetically resistant to *H. contortus* infection [22, 23]. Different studies have been carried out to define the underlying mechanism

of this resistance, and from these studies it appeared that the immune response plays an important role. The immunological response in these animals is mediated by the proliferation of mucosal mast cells, globule leucocytes and eosinophils [24-26], as well as specific antibody responses especially IgG1, IgM [27] and IgA [28, 29]. For the proliferation of these cells in the mucosal lining and for the specific antibody production, Th-cells appear necessary.

Treatment of infection

Treatment of a *H. contortus* infection involves the use of antihelminthic drugs such as benzimidazoles, levamisole and ivermectin [30]. Unfortunately, the poorly managed use of antihelminthic drugs has led to the continuing development of drug resistance to each successive new antihelminthic class [31]. Non-chemical control methods are required among which the most attractive is the production of a vaccine. Until now, attention has been mainly focussed on the identification of immunogenic proteins [11-13], but the possibility of carbohydrate antigens of the parasite acting as novel vaccine candidates has also been hypothesized [32-34]. Indeed there is a considerable body of evidence from studies of a variety of parasitic helminths that glycoconjugates are important in host parasite interactions. Indirect evidence from studies of lectin binding, sensitivity of antibody epitopes to periodate oxidation and susceptibility to peptide-N-glycosidase F (PNGaseF) [35] has indicated that many antigens of *H. contortus* are glycosylated. However, little is known about the nature of the glycans.

Schistosoma mansoni

Helminths from the Schistosoma species are the causative agents of human schistosomiasis, - also called bilharzia - , which is the main source of helminth infections inducing morbidity and mortality and is estimated to have 200 million people infected in 74 countries [36, 37]. It is found in Southwest Asia, South America, Africa and the Caribbean islands. The main species causing schistosomiasis are *Schistosoma mansoni*, *S. haematobium*, *S. intercalatum* and *S. japonicum* [38]. The different schistosoma subtypes have slightly different pathological characteristics.

S. mansoni has a very complex lifecycle (Figure 2). The helminth has both a sexual and an asexual reproduction stage and this is different from other trematodes, which are hermaphrodites. Infection of *S. mansoni* starts when larvae from the parasite, known as cercariae, are released by aquatic snails (snails of the genera *Biomphalaria*) and penetrate the skin of the mammalian host. Once in the host, the cercariae lose their tail thereby transforming into schistosomula and are transported to the portal circulation of the liver. In the portal circulation the schistosomula mature into adult male or female worms. The adult worms pair up and the females start producing eggs. The eggs that reach the wall of the intestine are secreted into the environment and mature into the second larval stage named miracidia. Miracidia have to find a fresh water snail within 8-12h and infect it. In the snail the asexual reproduction takes place. The sporocytes in the snail develop into cercariae and these migrate out of the snail into the fresh water reservoir to complete the lifecycle [39].

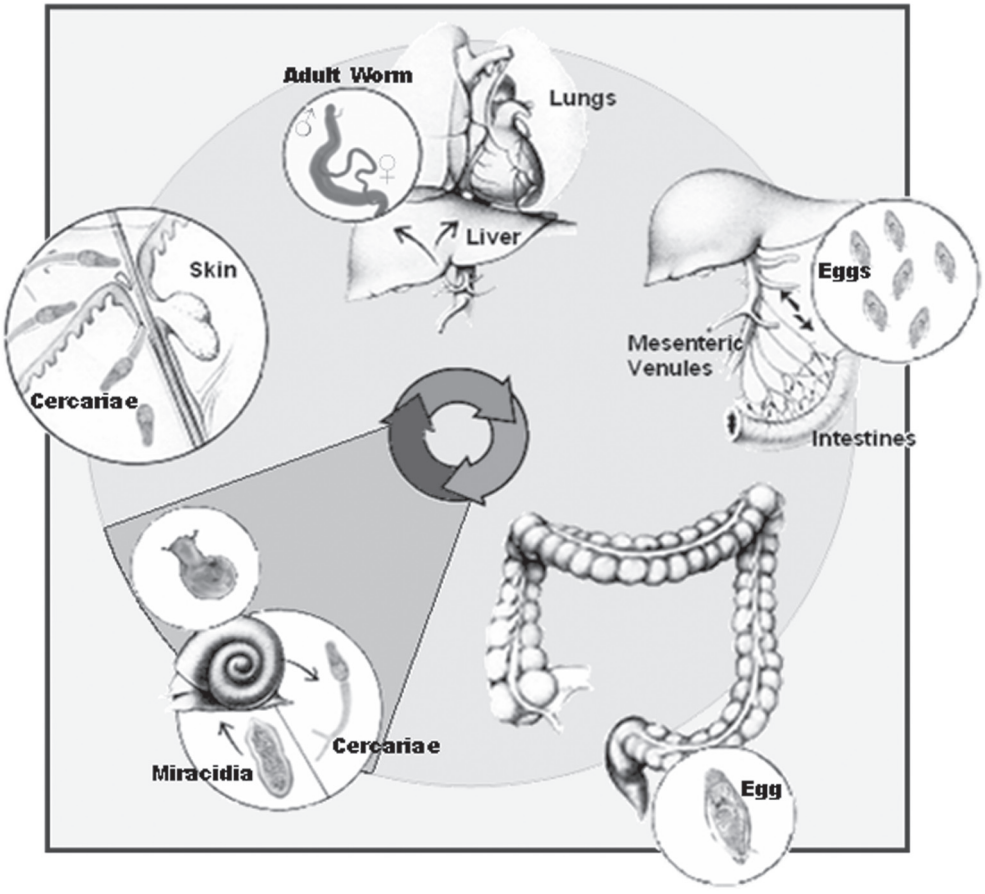

Figure 2: lifecycle *Schistosoma mansoni.*

Infection of *S. mansoni* starts when cercariae are released by aquatic snails (snails of the genera *Biomphalaria*) and penetrate the skin of the mammalian host. Once in the host, the cercariae transform into schistosomula and are transported to the portal circulation of the liver. In the portal circulation the schistosomula mature into adult male or female worms. The adult worms pair up and the females start producing eggs. The eggs that reach the wall of the intestine are secreted into the environment and mature into the second larval stage named miracidia. Miracidia infect the fresh water snail and in the snail the sporocytes develop into cercariae and these migrate out of the snail into the fresh water reservoir to complete the lifecycle.

Clinical course of *S. mansoni* infection

Infection by *S. mansoni* can manifest in two main clinical ways. The first is acute schistosomiasis, which is mostly prevalent in individuals that are not sensitised previously to the parasite. It manifests even before the appearance of eggs in the stool. The peak of the disease is generally around 6-8 weeks after infection. The human body responds to the infection with high disabling fever also known as Katayama fever [40, 41]. The immune response is characterized by an

upregulation of tumour-necrosis factor alpha (TNFα), interleukin-1 (IL-1) and IL-6, which are type 1 response cytokines [42]. During the progression of the disease, the development of egg-antigens induces a T-helper 2 (Th2) response and expression of pro-inflammatory mediators is downregulated [43]. These Th2 response inducing eggs are also the initiators of the second clinical condition namely the chronic state of schistosomiasis. Female worms, when matured, start producing up to thousands of eggs daily over a lifespan of 5-30 years [44]. About one-third of the eggs find their way back to the environment. The other two-third of the eggs fail to attach to the endothelium of the host and do not exit. These eggs get entrapped in the host tissue and within their 20 day lifespan in the tissue secrete numerous proteins, glycoproteins, glycolipids, and polysaccharides, which are highly antigenic [45, 46]. The immunological responses against these eggs cause the manifestation of schistosomiasis. Neutrophils and eosinophils start infiltrating the area around the eggs, which induces granuloma formation. The granulomas that are formed around the eggs block the micro-vascular blood supply and thus produce ischemic damage to the tissue leading to progressive scarring and dysfunction of the affected organ. This pathology of schistosomiasis is chronic and people with severe disease have low survival rates. Mortality mainly occurs via liver fibrosis and portal hypertension [39, 47].

Treatment of schistosomiasis

The drug most frequently used to treat schistosomiasis is Praziquantel (Biltricide, Droncit). An alternative drug is oxamniquine. Praziquantel has a serum half life of 0.8 to 1.5 h [48]. It is mainly metabolized by cytochrome P 450 pathway [49, 50]. Although the exact molecular mechanism of action of Praziquantel is not yet elucidated, it is shown that the early effects of Praziquantel are the induction of spastic paralysis of the worm musculature, which is accompanied and probably caused by a rapid Ca^{2+} influx inside the schistosome [51]. Another early effect is the change in morphology observed in the worm tegumentum, which is the outer layer of the worm. The morphological changes are initially represented by vacualization at the base of the tegumental syncytium and blebbing at the surface which leads to disruption of the tegument [52]. These morphological alterations are accompanied by the increased exposure of schistosome antigens at the parasite surface [53]. Although Praziquantel has the advantage of a single dose treatment, a major disadvantage is that it does not eradicate immature schistosomula. Infected people in a period of 3-5 weeks before drug application remain infected and re-infection is not blocked [8, 9, 54]. Moreover, resistance to Praziquantel has already been reported [55]. All these things taken together ask for a new medical approach for the treatment of schistosomiasis. Vaccines might provide a long-term solution to schistosomiasis, but further research is required as no vaccine is available yet.

Glycoconjugates in helminths

Upon infection, the host induces an inflammatory response to the invading helminth. However, most helminths are able to reside in the host for many years by evading clearance

of the immune system. Thus, the helminths must have developed mechanisms to evade the host immune system. Over the last few decades, more and more evidence has been emerging that glycoconjugates of the helminths play an important role in both the activation and the suppression of the immune system [15-17]. The glycosylation pattern of helminths changes during the different life stages and this may contribute to the different responses to the various life stages of the worms. Glycoconjugates consist of a broad range of oligosaccharides, covalently linked to lipids (glycolipids) or to proteins (glycoproteins). Helminth glycoproteins are built up similarly as those of mammals and can be classified into two groups based on the type of linkage of the glycan to the protein: Asn-linked (N-linked) and linked to Ser or Thr (O-linked).

Helminth glycoproteins

An oligosaccharide linked to an amide group of asparagine (Asn) is called an **N-linked glycan or N-glycan**. The first step in the formation of an N-glycan is the assembly of an oligosaccharide structure on the lipid Dol-P on the cytosolic side of the endoplasmatic reticulum (ER). The N-glycan precursor consists of mannose 5- glucosamine 2 (Man5GlcNAc2)-Dol, which 'flips' across the membrane bi-layer to end up in the ER. The Dol-p precursor is further elongated with 4 mannoses and 3 glucose (Glc) residues, after which the Glc3Man9GlcNAc2-Dol precursor is transferred onto an Asn in an Asn-X-Ser/Thr (X can be any amino acid except Pro [56]) sequence of a newly translated protein. The oligosaccharide chain is then trimmed down to Man8GlcNAc2-Asn. In the Golgi, the high mannose type N-glycan can take two pathways. One involves the phosphorylation of the N-glycan, which targets the N-glycan towards the lysosomes. The other involves further trimming of the glycan by mannosidases to Man5GlcNAc2-Asn, which is the precursor for further diversification. The Man5GlcNAc2-Asn structure formed in this process is the precursor of the three major N-glycan types: High-mannose-type, Complex-type and Hybrid-type [57, 58] (Figure 3).

Helminth parasites and their mammalian hosts use similar mechanisms of N-glycosylation, although their terminal glycosylation profiles generally differ. Helminths show glycan modifications, which are not found or uncommon within their mammalian host, and vice-versa. For example, sialic acids are generally found in vertebrates but are lacking in helminth glycans. Possibly, helminths lack the genes involved in the sialic acid synthesis [16]. Instead, fucose seems to be abundantly present in helminth glycans, in particular in *S. mansoni*. Differences are found both in modification of the core structures and the antennae of the glycans. The proximal GlcNAc of the N-glycan core of schistosome adult worms and eggs can contain an α3-linked or α6-linkage fucose [59]. Such modification by fucose of the N-glycan core has also been found in *H. contortus*. Remarkably, in *H. contortus* also core-fucosylation of the distal GlcNAc has been demonstrated, which so far has not been found in any other helminth [35, 60]. In addition, the N-glycan core of *S. mansoni* cercariae and eggs can be modified by a β2-linked xylose, a modification also found in plants [61] but not in mammals [62]. The antennae of N-glycans of mammalian species mostly consist of Galβ1-4GlcNAc (LN, LacNAc) backbone structural units but many helminth species express instead, or in addition to LN units,

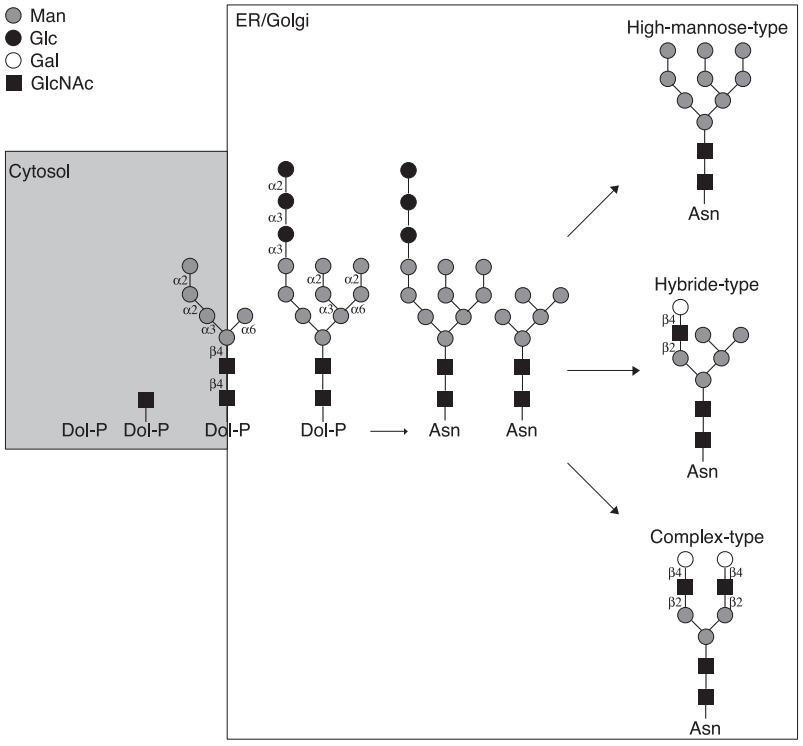

Figure 3: N-glycosylation.

N-glycosylation is initiated by the formation of an oligosaccharide assembly on a lipid Dol-P on the cytosolic side of the ER. The Man5GlcNAc2 'flips' across the membrane bi-layer to end up in the ER. The Dol-p precursor is further elongated with 4 Man and 3 Glc and then transferred onto an Asn. After further processing, the Man5GlcNAc2-Asn precursor is formed and this structure is further modified into the three major N-glycans found in vertebrates: High-mannose-type, which only has Man residues connected to the core; Hybrid-type, which only has Man residues connected to the Man-6 arm of the core, and the Complex-type, which generally has one or two antennae both on the Man-6 and Man-2 arm of the core, and or core-modifications (not shown).

GalNAcβ1-4GlcNAc (LDN, LacdiNAc) and/or GlcNAcβ1-4GlcNAc (chitobiose) units [63, 64]. Many different terminal glycan antigens can be discriminated within N-glycans, O-glycans and the glycolipids. In *S. mansoni* these structures include the glycan antigens Le^X (Lewis X, Galβ1-4(Fucα1-3)GlcNAc), poly Le^X, LDN, fucosylated LDN (including Fucα1-3GalNAcβ1-4GlcNAc (F-LDN) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF)) or multi fucosylated LDN, which are differentially expressed in the life stages of *S. mansoni* [17]. LDNF antigens are also demonstrated in *H. contortus* [34] (Figure 4).

In principle, there are many types of **O-glycosylation**, but this dissertation will only refer to the common mucin-type of O-glycosylation. O-glycans or O-linked oligosaccharide formation starts when a GalNAc residue is linked to the OH-group of a serine (Ser) or threonine (Thr).

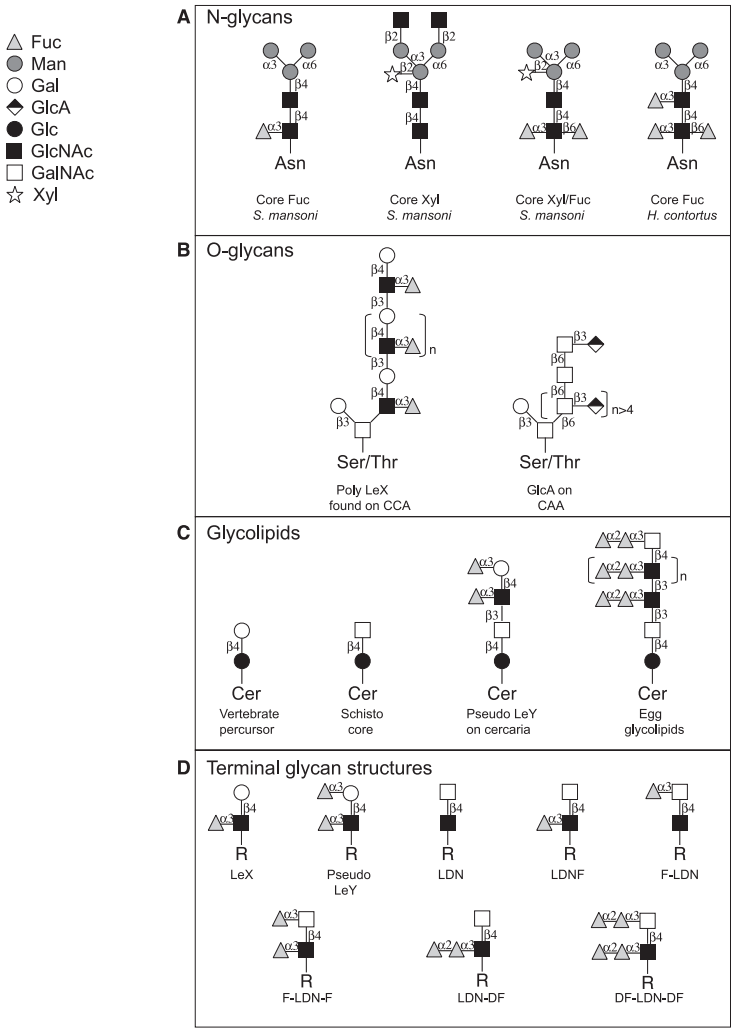

Figure 4. Examples of helminth glycan structures.

A.) N-glycan core structures of *S. mansoni* cercariae and egg can be modified by β2-linked Xyl, and egg glycoproteins express truncated N-glycan core structures carrying α3-linked Fuc in addition to β2-linked Xyl. N-glycan core fucoses are also found in *H. contortus*. B.) Examples of O-glycans are schistosome circulating cathodic antigens (CCA) and circulating anodic antigens, with respectively poly Le^X and GlcA. C.) Schistosome glycolipids are built up on a GalNAcβ1-4Glc-Cer core (Schisto-core). Glycolipids of cercariae contain unusual pseudo Le^Y structures, and egg glycolipids have been reported to be heavily fucosylated. D.) Examples of terminal glycan structures found on *S. mansoni* glycoconjugates. Some of these structures are also (not commonly) found in the mammalian host like LDN, LDNF and Le^X. Other structures like pseudo Le^Y, F-LDN, F-LDN-F, LDN-DF and DF-LDN-DF are not found in mammalian glycans. LDNF structures are also demonstrated in *H. contortus*.

In contrast to N-glycan formation, O-glycan formation is not dependent on a lipid linked oligosaccharide precursor. In mammals, the initial GalNAc can be elongated with a GalNAc, a GlcNAc or Gal, thereby creating different core structures. Four common core structures have been identified in mammals. The core 1 structure is the precursor of the core 2 structure and the core 3 structure is the precursor for the core 4 structure. These core structures can subsequently be further elongated with Gal, GalNAc, GlcNAc, fucose, or N-acetylneuraminic acid (NeuAc), resulting in a great variety of O-glycans. The terminal ends of the O-glycan chains can be similar to those of N-glycans, however the O-glycans are generally less branched [58] (Figure 5).

In schistosomes, O-glycans can consist of a single O-linked GlcNAc residues, or short mucin-type disaccharides on glycoproteins [65, 66]. However, O-glycans of schistosomes can also be large multi-fucosylated structures as expressed in the glycocalyx of cercariae [67]. They have been described to contain high amounts of LDN- and LN-based structures, which can be heavily fucosylated. The glycoproteins that are shed from the worms gut contain a large number of O-linked glycans, namely cathodic and anodic antigens (respectively CCA and CAA). The O-glycans of CCA mainly contain long linear multimers of Le^X trisaccharides [68]. CAA are constructed of a repeating β1-6- GalNAc backbone with β1-3 linked GlcA side chains [69] (Figure 4). Until now, little is known about the O-glycosylation of *H. contortus*.

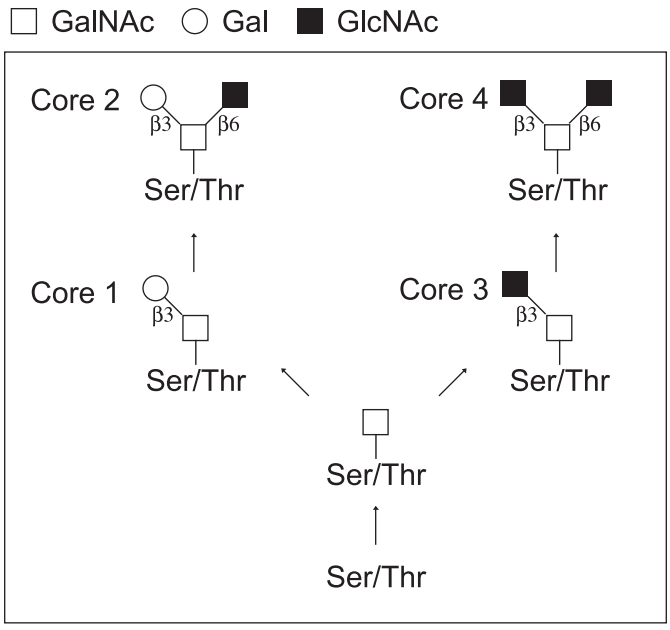

Figure 5. Biosynthesis of O-glycan core structures.
The initial step of O-glycosylation is the addition of a GalNAc to a Serine or a Threonine of a glycoprotein. Addition of a β1-3-linked Gal or β1-3-linked GlcNAc results in the formation of core 1 and core 3, respectively. Core 2 and core 4 are formed by the addition of a GlcNAc.

Helminth glycolipids

The most prevalent glycolipid structure in mammals is the glycosphingolipid, which is also widely expressed in helminths. In vertebrates, they are ubiquitous in the plasma membrane and modulate membrane protein function, facilitate cell-cell recognition and play a role in host pathogen interactions. Glycosphingolipids are formed by the linkage of a Gal or a Glc to a terminal hydroxyl group of a ceramide (Cer) creating a GalCer or GlcCer, which occurs on the cytosolic side of the Golgi-ER apparatus. The elongation of the precursor structures GalCer and GlcCer occurs on the luminal side of the Golgi. This indicates that the precursors need to 'flip' to the luminal side where further elongation will take place. The number of extensions on GalCer is somewhat limited but GlcCer gives rise to a broader range of extensions. In vertebrates, a Gal is subsequently linked (β1-4) to the GlcCer precursor, creating lactosylceramide. The molecule can be elongated further in a stepwise fashion leading to a wide variety of structures. Most of the glycosphingolipids derived from GlcCer are divided into three classes: the ganglio-series, the lacto/neolacto-series and the globo/isoglobo-series [58].

In schistosomes a similar GlcCer precursor has been demonstrated as found in vertebrates, but in contrast to the addition of a Gal to the GlcCer schistosomes add a GalNAc in a β1-4-linkage. The resulting structure, GalNAcβ1-4GlcCer, is known under the name Schisto-core [70, 71]. The glycolipids of schistosomal cercariae and eggs have been characterized in detail, however less structural details are reported about glycolipids of adult worms. The elongations of the Schisto-core can be similar to the structures on O- and N-glycoproteins like F-LDN and F-LDN-F. However, schistosomes also express characteristic glycolipid structures. Schistosome egg glycolipids can contain for example 4 repeats of (Fucα1-2Fucα1-3)GlcNAc terminating with a (Fucα1-2Fucα1-3)GalNAc [59], the first Fuc being optional in both cases. In cercariae glycolipids Le^X and Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (Pseudo Le^y) structures have been demonstrated [17] (Figure 4). Stage specific expression patterns are not only found for the glycan part of the glycolipids in *S. mansoni* but also for the ceramide part of mono- and dihexosides. The dominant ceramide monohexosides fatty acid in the three life stages is hydroxylated palmitic acid (C16h:0) but egg ceramide did not contain fatty acids with more than 20 carbon atoms while adults have ceramide parts with 24 carbon atoms. Cercarial ceramide monohexides even have fatty acids varying form 24 to 28 carbon atoms [72]. To date, no reports describe the glycolipid composition of *H. contortus*.

Biosynthesis of glycans by glycosyltransferases and glycosidases

A huge variability exists in the amount of glycan structures on glycoconjugates as a result of the various ways monosaccharides can be linked to each other. The enzymes catalyzing the elongation and trimming of the glycan chains are known as glycosyltransferases and glycosidases.

Different from other biological macro-molecules such as polypeptides, synthesized by a template-driven mechanism, the synthesis of an oligosaccharide is mediated by the

sequential action of various glycosyltransferases. These enzymes are specific for the added monosaccharide, the linkage that is formed and the acceptor structure onto which the monosaccharide is linked. Although there is a large number of glycosyltransferases, they all catalyse a glycosidic binding. The catalytic reactions involve the transfer of a monosaccharide from a high-energy nucleotide sugar donor (e.g. UDP-Gal), to an acceptor molecule, which can be a monosaccharide, oligosaccharide or non-glycan moiety (Figure 6).

Until now, only few helminth glycosyltransferases have been identified. Although some glycosyltransferase activities have been identified in *H. contortus* and *S. mansoni* [73-76], no recombinant forms of these enzymes are available to use for enzymatic synthesis of helminth glycans. However, several glycosyltransferases from the free-living nematode *Caenorhabditis elegans* have been identified and cloned, such as the in eukaryotic cells highly preserved mannose specific β1,2-GlcNAc transferase [77], several fucosyltransferases [78-80], a β1,4-Gal-transferase [81] and a β1,4-GalNAc transferase [82]. As described in **Chapter 3**, a recombinant form of this latter glycosyltransferase has been used for the production of LDN.

During the process of N-glycosylation, trimming of specific intermediate oligosaccharides occurs by the action of glycosidases, which can be classified into endo-and exo-glycosidases. Endo-glycosidases catalyze the cleavage of an internal glycosidic bond in an oligosaccharide. Exo-glycosidases cleave off end-standing monosaccharides. In general, glycosidases catalyse the hydrolysis of a specific glycosidic linkage [58].

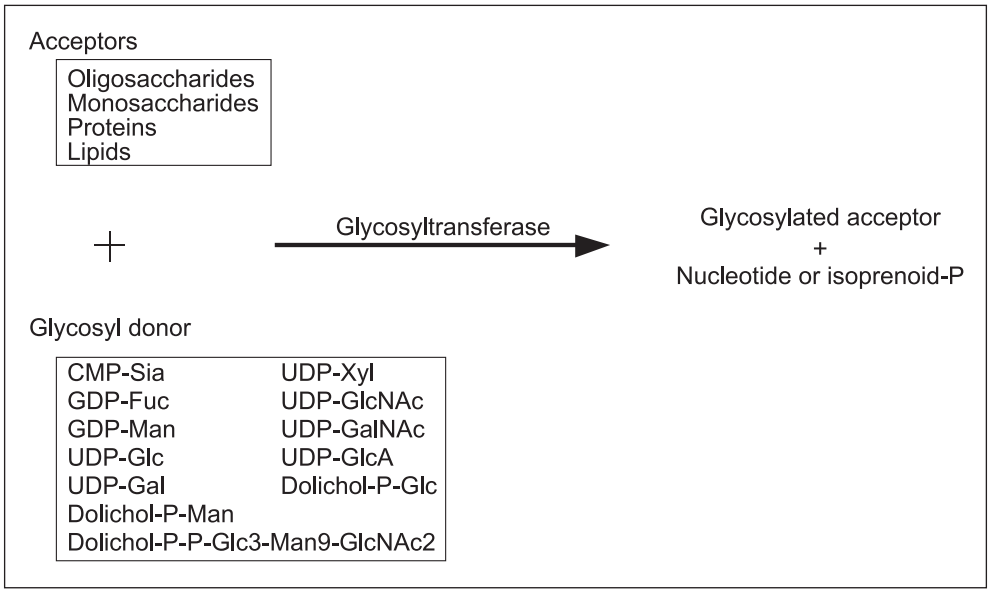

Figure 6. Glycosyltransferases catalyze a glycosylation reaction.
A glycosyltransferase uses a glycosyl donor and an acceptor substrate to catalyze the initiation or elongation of a glycan structure.

Host immune responses upon helminth infection

Helminth infection is typically characterized by the induction of a type 2 response, which includes the activation of eosinophils, basophils and mast cells, the production of IgE, and the proliferation of Th2 cells that secrete IL-4, IL-5, IL-9, IL-10 and IL-13. In contrast, type 1 responses are typically evoked by bacteria and viral infections. A type 1 response is characterized by interferon-γ (IFN-γ) producing Th1 cells, neutrophils and classically activated macrophages [5, 83-85].

Although the immune response elicited by helminth infection is predominantly type 2, the immune response is not solely directed to destroying the parasite. In helminth infections two other processes have to be kept in mind. Firstly, helminth eggs and larvae migrate in the body and thus induce tissue damage. Therefore an appropriate response to the pathogen may include containment and tissue repair. This repair mechanism must be kept under control to prevent destructive tissue remodelling and fibrosis. Secondly, the immune response against helminth infections is not merely a type 2 response, but includes anti-inflammatory components. The type 2 response is a reflection of the host immune system recognizing the helminth, while the anti-inflammatory component is likely to reflect the parasite's adaptation to evade the immune system. This type 2 response is called a modified or regulatory type 2, which is characterized by diminished IL-5 and IL-13 secretion and increased IL-10 and/or transforming growth factor-β (TGF-β), and in many cases parasite-specific immune suppression [7, 85].

In mammals the adaptive and the innate immune system work together to produce an adequate immune response against invading pathogens like helminths. The adaptive immune response is characterized by the activation of B- and T-cells. When T- and B-cells recognise the antigen, for which they are specific, they start to clonally expand and induce immunological memory for these antigens. However, the disadvantage of this system is that it is not able to recognize the antigen by itself for the initiation of this process. A second drawback of the adaptive immune system is the time it needs to differentiate into effector cells, which is at least 4-7 days. This is too long for a quickly replicating, or morphologically changing pathogen. Fortunately, the adaptive immune system works side by side with the faster, less fine-tuned innate immune system.

The innate immune system is evolutionarily preserved. It acts as a pathogen sensor, provides the first line of defence and initiates and determines the function of the adaptive immune response. The functioning of the innate immune response is dependent on recognition of conserved molecular patterns on the pathogen, called pathogen-associated molecular patterns (PAMPs). The reasons why these PAMPs are good targets for the innate immune recognition are: PAMPs are not expressed by the host, are conserved in subsets of microorganisms and generally are thought to be essential for the survival of the pathogen. These antigenic molecules are recognized by an array of receptors called Pathogen/Pattern recognition receptors (PRR). The principle functions of PRR include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways and

induction of apoptosis. PRR are expressed on the cell surface, intracellular compartments, or secreted into the bloodstream and tissue fluids. Classification can be made on their signalling capacity. Non-signalling and signalling PRR are described. The non-signalling PRR include transmembrane and soluble factors. The soluble molecules include active phase proteins such as C-reactive proteins and soluble lectins. When bound to the antigen, the antigen becomes vulnerable for phagocytosis and/or recognition by the complement system. Transmembrane non-signalling molecules bind the pathogen, facilitate the internalization and direct the pathogen to the lysosomes. These transmembrane molecules do not need a signalling cascade to induce this function. Various immune cells in addition express signalling PRR. Besides the recognition of the pathogen, they are able to trigger a signalling pathway, which leads to the production of (anti) inflammatory cytokines or type I IFNs. The signalling PRR include cytosolic and transmembrane molecules. Examples of cytosolic PRR are the nucleotide-binding oligomerization domain (Nod) molecules, Retinoic acid inducible gene I (RIG-I) and Mda-5. Important transmembrane signalling PRR are Toll-like receptors (TLRs) and some C-type lectins.

Toll-like receptors

One of the best studied family of PRR is the TLR family. TLRs are broadly expressed by immune cells like dendritic cells, macrophages, neutrophils, eosinophils but also on endothelium, epithelium and keratinocytes [86]. The primary function of the TLRs is to sense the presence of pathogens and to induce an appropriate immune response. TLRs recognize PAMPs, which can be "foreign" lipids, proteins, carbohydrates or nucleic acids. Mammals express at least 10 TLRs [87-89]. In humans, some TLRs are found on the cell surface (TLR-1,2,4,5 and 6) whereas others reside within the endosomal compartments (TLR-3,7,8,9). The endosomal TLR-ligand binding requires internalization of the pathogen before signalling is possible. TLRs are expressed as homodimers or heterodimers. Some TLRs require accessory proteins to recognize their ligand. For example, TLR4 recruits several adaptor molecules to mediate endotoxin recognition. Lipopolysaccharide-binding protein (LBP) accelerates the binding of endotoxins, like Lipopolysaccharide (LPS), to CD14. CD14 is a membrane bound molecule that does not signal by itself, but facilitates the transfer of LPS to MD2 which in turn facilitates the LPS recognition by TLR4 [90] (Figure 7).

TLRs are type I transmembrane receptors consisting of an extracellular leucine-rich repeat (LRR) domain, which facilitates the pathogen recognition and an intracellular Toll/IL1 receptor domain (TIR) that is essential for signal transduction. All members of the TLR family have similar intracellular TIR domains and consequently signal in a similar manner. Basically, two pathways are described in TLR signalling namely the Myeloid differentiation factor 88 (MyD88) dependent and the MyD88 independent pathway. In the MyD88 dependent pathway, MyD88 transfers the signal to IL-1 RI-associated protein kinases (IRAK), which transport the signal to other transfer proteins leading to transcription of nuclear factor-kappa B (NF-κB) and other nuclear factors. The MyD88 independent pathway transfers the signal directly

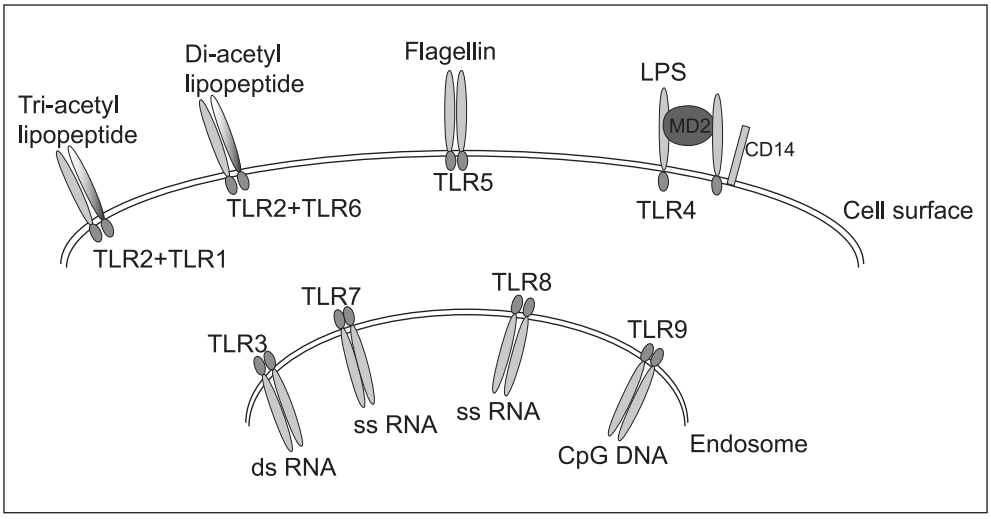

Figure 7. Membrane and endosomal expressed TLRs
TLR1, TLR2, TLR4, TLR5 and TLR6 are TLRs expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular compartments

onto TIR domain-containing adaptor-inducing IFN (TRIF), which transfers the signal to other transport proteins also ending up in the transcription of NF-κB. The transcription of NF-κB and other transcriptional factors, induced by the different TLR signals, leads to the production of inflammatory cytokines [86].

Several helminth-derived structures are reported to trigger activation of TLRs. Glycoconjugates containing Lacto-N-fucopentaose III (LNFPIII, a milk sugar carrying a Le^x moiety) induce signalling in murine dendritic cells (DCs) via TLR4, suggesting a role for Le^X in the induction of Th2 responses [91]. However, a recent report indicates that in mice the induction of a Th2 response by schistosome SEA, which also include Le^X antigens, is independent of MyD88, TLR2 and TLR4 [92]. Clearly, much has still to be understood about the molecular mechanism involved in the induction of Th2 responses by schistosoma antigens. Other schistosoma antigens have been described to trigger TLRs. TLR2 activation has been demonstrated by lysophosphatidylserine (Lyso-PS)-containing molecules from *S. mansoni* eggs and TLR3 can be stimulated by dsRNA from *S. mansoni* eggs [93]. Furthermore, excretory/secretory (ES)-62, a phosphorylcholine-containing protein secreted by *Acanthocheilonema viteae*, contains TLR4 ligands that induce DCs to skew naïve CD4 T-cells towards a Th2 response [94].

C-type lectins

C-type lectins are defined by their ability to bind carbohydrates in a Ca^{2+}dependent manner. Soluble and membrane bound proteins are included in this family. The family members share a similar carbohydrate recognition domain (CRD), which consists of 18 highly conserved amino acid residues including two-folds of disulfide bonds, formed by four cysteine residues. A wide

variety of C-type lectins are expressed on the cell surface of phagocytic cells like macrophages and DCs.

C-type lectins have a broad range of functions. They facilitate cell adhesion, cell-cell contact by endogenous ligands, and can be involved in the recognition of pathogens and in this respect resemble TLRs. C-type lectins have endocytic functions and have been reported to internalize pathogens and direct them to the lysosomes. In addition, some C-type lectins have also been reported to have signalling motives thereby enabling them to induce cell signalling. It has become clear that TLRs and C-type lectins co-operate to facilitate an appropriate immune response.

C-type lectins expressed on the membrane of dendritic cells are either type I transmembrane or type II transmembrane proteins (Figure 8). Type I C-type lectins include the MR (CD206)

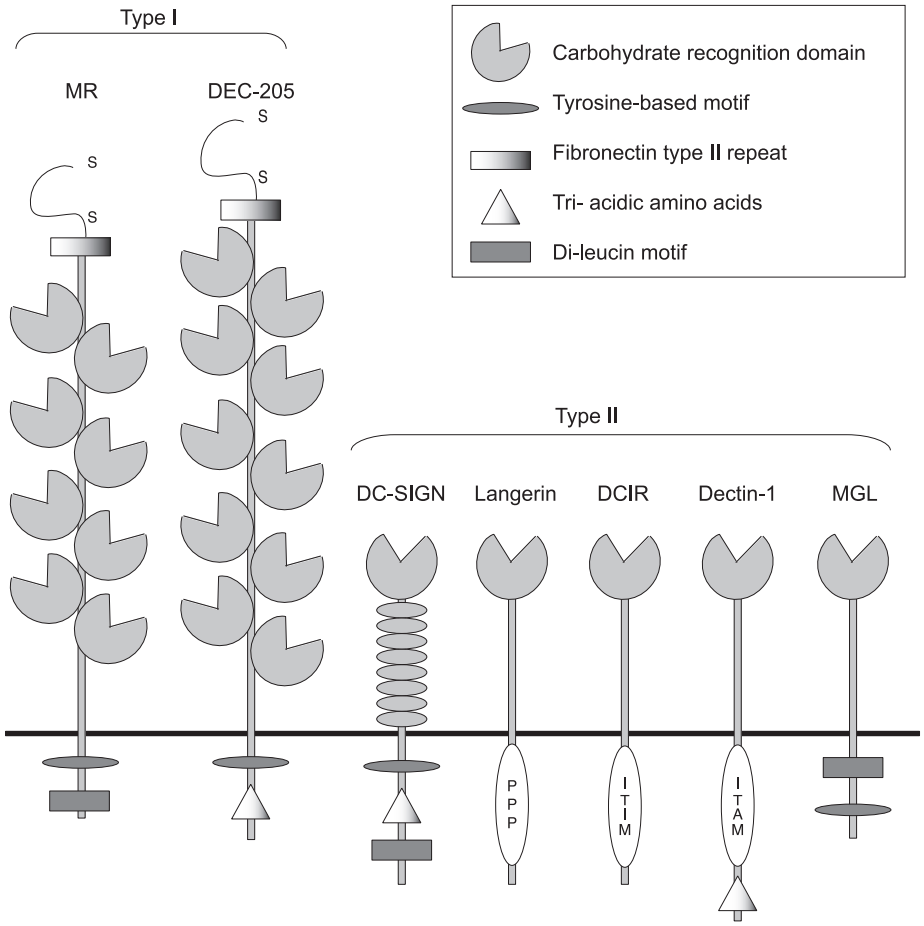

Figure 8. Two types of C-type lectins.
C-type lectins can have one or multiple carbohydrate recognition domains, as demonstrated in this figure showing type I C-type lectins, like DEC 250 and MR, and type II C-type lectins.

and DEC-205 (CD205). Most of the group members have an N-terminal cysteine-rich domain and 8 to 10 CRDs. The CRDs can be fully or partially conserved, the latter lacking the ability to bind Ca^{2+} or carbohydrates. The type II C-type lectins include macrophage galactose-type C-type lectin (MGL, CD301), DC-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), Langerin (CD207) and the members of the DC immunoreceptor (DCIR) and DC-associated C-type lectin (Dectin)-1. Many of these receptors are involved in the recognition and internalization of pathogens and by this way facilitate the processing of the pathogen and expression onto MHC molecules on APC. Although the endocytic capacities are similar, the delivery to the lysomal compartment can be different. MR delivers antigens to the early endosomes and recycles to the cell surface, DEC 205 and DC-SIGN on the other hand recycle the antigen to the late endosomes or lysosomes where they are degraded together with the antigen [95].

The first components of helminth pathogens recognized by the host during helminth infections will be the outer surface or secreted products of the parasite, which are mostly heavily glycosylated. This suggests that C-type lectins that recognize glycan antigens, are ideal receptors to detect the presence of helminths. Until now, only a few reports have been dedicated to this topic. It has been reported that L-SIGN recognizes soluble egg antigens (SEA) of *S. mansoni* [96]. SEA also interact with DC-SIGN, the MR and MGL and induce a Th2 response [97]. Next to the recognition of Le^X and LDNF antigens of SEA by DC-SIGN, DC-SIGN also recognizes Le^X and pseudo-Le^Y (Fucα1-3Galβ1-4(Fucα1-3)GlcNAc) on cercariae of *S. mansoni* [98]. It is proposed that the interactions of dendritic cell C-type lectins with schistosome glycans play a role in modulation of dendritic cell function, however these functions and the molecular mechanisms are virtually unknown.

While the main function of C-type lectins is to internalize antigens for degradation in order to enhance antigen processing and presentation, TLRs recognize foreign structures and trigger intracellular signalling cascades that lead to the production of pro-inflammatory cytokines, thus causing T–cell activation. But an increasing body of evidence suggests that TLRs and C-type lectins communicate with each other, and cross talk is critical for the balance between immune tolerance and immune activation. The C-type lectin Dectin-1 has been reported to act together with TLR2 to enhance the production of TNFα, and facilitates Th1 skewing [99]. Mycobacterial component ManLam is recognized by DC-SIGN, blocks LPS induced upregulation of maturation markers CD80, CD86, CD83, HLA-DR, secretion of IL-12 and induces the production of IL-10, an immunosuppressive cytokine [100]. Similar results were found with the triggering of DC-SIGN by Lewis structures of *Helicobacter pylori*, which induce secretion of IL-10 and suppression of the Th1 response [101]. In contrast, a mutant LPS from *Neisseria meningitides (lgtB)* has been reported to induce a Th1 response in DC via DC-SIGN signalling [102]. These reports indicate that TLRs and C-type lectins work together to orchestrate an adequate immune response, which may vary dependent on the specific structures of the antigens encountered.

Galectins

Another family of glycan recognition molecules playing an important role in innate immunity are the galectins. Galectins, formerly known as soluble-type lectins (S-type or S-Lac), are a broad conserved group of lectins in mammalians. They have been identified in a highly diverse range of organisms from vertebrates to insects, nematodes and fungi. A common characteristic of the galectins is the presence of at least one CRD of about 135 amino acids with an affinity for β-galactoside. In mammals 14 galectins have been found until now. According to the architecture of the lectins, they are divided into 3 types: Chimera-type, proto-type and tandem repeat-type. The proto-type consists of a single CRD (galectin-1, -2, -5, -7, -10, -11, -13 and -14). The tandem repeat-type consists of two CRDs connected by an unconserved linker region (galectin-4, -6, -8, -9 and -12) and the chimera–type consists of a single CRD and is extended with an N-terminal domain (galectin-3). Galectins are expressed both intracellularly and extracellularly [103, 104]. Although expressed extracellularly, galectins do not have a signalling sequence for transport into the ER and are not glycosylated, although some galectins contain N-glycosylation sites, indicating that they are not secreted via the classical secretory pathway [105] (Figure 9).

Galectin-3 (Gal-3) is one of the best studied members of the galectin family and the only chimera type lectin known until now. It is composed of a C-terminal CRD and a N-terminal domain consisting of multiple PGAYPG repeats involved in multimerization. Gal-3 is expressed by a variety of immune cells like T-cells [106, 107], eosinophils [108], dendritic cells [109] and monocytes/macrophages [110].

Gal-3 has been associated with a tremendous amount of different functions. For example, extracellular Gal-3 has been connected to an increased apoptosis in T-cells [111]. Intracellular Gal-3 on the other hand has been reported anti-apoptotic in B-cells, T-cells and macrophages and this is presumed to be related to the engagement in the apoptosis-regulation pathway and/or modulating mitochondrial homeostasis. Next to the influence on apoptosis, Gal-3 has also immune modulatory properties as it can induce the degranulation of neutrophils and

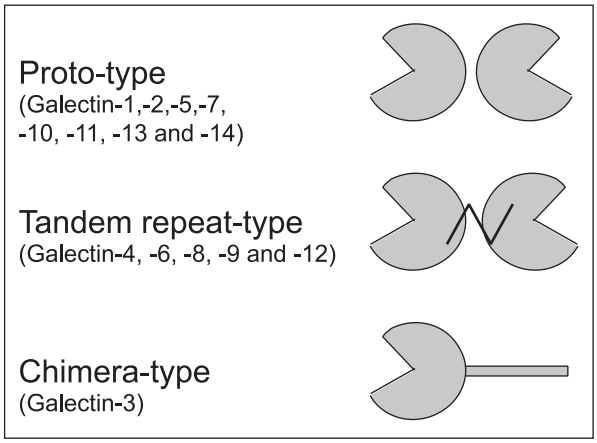

Figure 9. Galectins.
Proto-type galectins, like galectin-1, -2, -5, -7, -10, -11, -13 and -14, consist of a monomer or a dimer with two identical CRDs. Tandem repeat-type, like galectin-4, -6, -8, -9 and -12, cross-link two different CRDs. Chimera-type lectins like galectin-3 contain 1 CRD and an extended N-terminal domain.

mast cells and has positive effects on chemotaxis and phagocytosis by macrophages [103]. On the other hand, a negative effect has been reported on the production of IL-5 in eosinophils, IL-12 production in dendritic cells and differentiation of B-cells into plasma cells. The IL-1 production in monocytes, IL-2 production in T-cells and the IL-8 production in neutrophils is increased in the presence of Gal-3 (Figure 10). Gal-3 has also been associated with host-parasite interactions. It recognizes LDN and LN structures expressed on *S. mansoni* eggs [112].

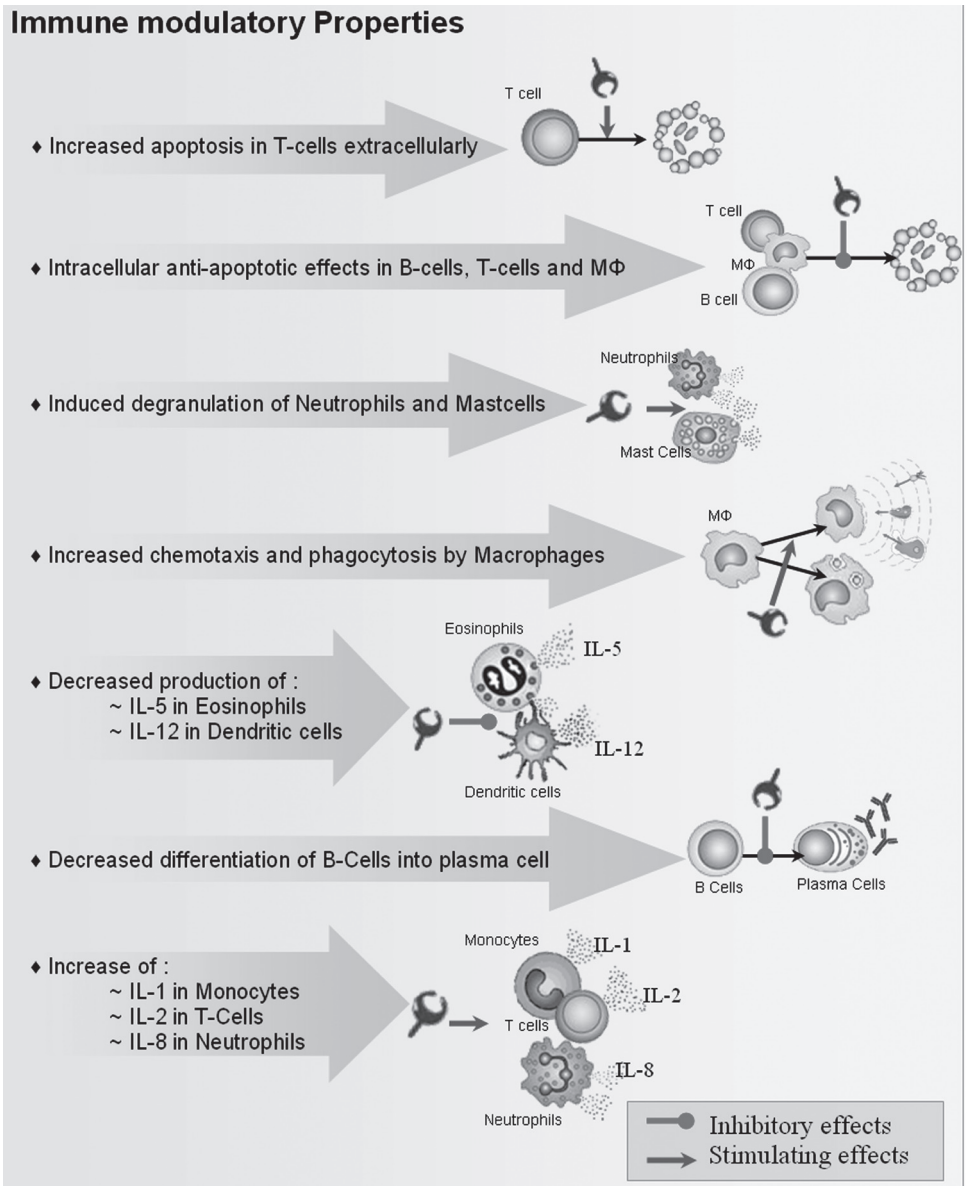

Figure 10 Immunological functions of galectin-3.

Gal-3 appears to specifically recognize structures of the protozoa parasite, *Leishmania major,* which may lead to the increased survival of *L. major* [113]. *Trypanosoma cruzi* seems to utilise Gal-3 to adhere to the extracellular matrix via surface glycoprotein recognition [114]. This could indicate that parasites manipulate the recognition of surface glycoconjugates by galectins to facilitate their survival. Infection of Gal-3 knockout mice resulted in a more pronounced Th1 response than infection of their wild type counterparts, which may indicate a role for Gal-3 in inducing a Th2 response [115]. In conclusion, the role of Gal-3 in host parasite infections is far from clear. One of the aspects complicating the study of Gal-3 function in immune regulation, is that extracellular galectins may act on other cells than the cells that produce and secrete them, and their regulation of expression and secretion is virtually unknown. This aspect of Gal-3 biology has been studied in dendritic cells, being important cells regulating the T helper cell response, and the results are described in **Chapter 4** of this thesis.

Outline of this dissertation

This dissertation highlights the molecular interaction of helminth glycans with the host's immune system, and its capacity to modulate the immune response. Two different helminths are used, namely the gastrointestinal nematode *Haemonchus contortus* (**Chapter 2**) and the trematode *Schistosoma mansoni* (**Chapter 5**). In **Chapter 2** we show that serum of lambs vaccinated with ES proteins of *H. contortus* contains immunoglobulin G (IgG) antibodies that recognize the glycan antigen Galα1-3-GalNAc-, in addition to GalNAcβ1-4(Fucα1-3)GlcNAc- (LDNF), which was reported previously by Vervelde et al [34]. Analysis by anti-glycan antibodies also revealed that *H. contortus* glycoproteins contain both Galα1-3-GalNAc-, and Galα1-3-Gal structures. **Chapter 3** describes a method to synthesize the helminth glycans GalNAcβ1-4GlcNAc (LDN) and α3-fucosylated LDN (LDNF), as a first approach to generate defined helminth glycans in order to study their interaction with the immune system. In **Chapter 5** we show that *S. mansoni* worm glycolipids induce maturation of DC and induce a T helper 1 (Th1) response. The data suggest that worm glycolipids activate Toll-like receptor 4 (TLR4) to induce a proinflammatory response. Remarkably, activation of TLR4 requires the interaction of the worm glycolipids with the C-type lectin DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) that recognizes a subfraction of worm glycosphingolipid species exposing Galβ1-4(Fucα1-3)GlcNAc- (Le^X) and LDNF glycan antigens. In addition to DC-SIGN, the host lectin galectin 3 (Gal-3), plays an important role in interaction with helminth glycans. In **Chapter 4**, the regulation of expression of Gal-3 in dendritic cells is described.

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CHAPTER 2

Vaccination-induced IgG response to Galα1-3GalNAc glycan epitopes in lambs protected against *Haemonchus contortus* challenge infection

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Abstract

Lambs vaccinated with *Haemonchus contortus* excretory/secretory (ES) glycoproteins in combination with the adjuvant Alhydrogel are protected against *H. contortus* challenge infection. Using glycan microarray analysis we showed that serum from such vaccinated lambs contains IgG antibodies that recognize the glycan antigen Galα1-3GalNAc-R and GalNAcβ1-4(Fucα1-3)GlcNAc-R. Our studies revealed that *H. contortus* glycoproteins contain Galα1-3Gal-R as well as significant levels of Galα1-3GalNAc-R, which has not been previously reported. Extracts from *H. contortus* adult worms contain a galactosyltransferase acting on glycan substrates with a terminal GalNAc, indicating that the worms possess the enzymatic potential to synthesize terminal Gal-GalNAc moieties. These data illustrate that glycan microarrays constitute a promising technology for fast and specific analysis of serum anti-glycan antibodies in vaccination studies. In addition, this approach facilitates the discovery of novel, antigenic parasite glycan antigens that may have potential for developing glycoconjugate vaccines or utilization in diagnostics.

Keywords: *Haemonchus contortus*, *Toxocara canis*, Antigenicity, Glycosylation, α-galactose, Carbohydrate, Glycan microarray

Introduction

Infections by gastro-intestinal nematodes are wide-spread and cause substantial damage, both in terms of well-being of livestock and economic losses by farmers. *Haemonchus contortus* is a common gastro-intestinal nematode, which resides in the abomasum of sheep and feeds on the host's blood. Treatment by antihelminthic drugs is an effective way to control infection, although increasing drug resistance requires another and urgent approach to combat these infections (Jackson and Coop, 2000). Many studies focussed on the identification of immunogenic protein antigens of *H. contortus* and the analysis of their potential to induce protective immunity by vaccination (Vervelde et al., 2002; Knox et al., 2003; Redmond and Knox, 2006). Several native antigens, including "hidden" gut-derived antigens, can induce protection against *H. contortus* (Knox et al., 2003). However, attempts to induce protection employing recombinant forms of these antigens are not encouraging, suggesting that specific post-translational modifications, such as glycosylation, may contribute to the protective properties of these proteins (Vervelde et al., 2002).

Glycosylation can greatly contribute to the immunogenicity of proteins, especially when the glycans are foreign to the host. Glycans are abundant on the surface and secretory products of helminths, and are well exposed to the environment. Both glycans of the parasitic trematode *Schistosoma mansoni* (Okano et al., 1999, 2001) and nematode-glycans (Tawill et al., 2004) have the capacity to trigger T-helper 2 (Th2) type responses and the production of glycan-specific antibodies in their hosts (Okano et al., 1999, 2001). Individuals infected with *Schistosoma* species and chimpanzees immunized with radiation-attenuated cercariae showed high levels of anti-glycan serum IgG to the glycan antigens GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) and Fucα1-3GalNAcβ1-4GlcNAc (F-LDN), glycan motifs that are not found in mammals (van Remoortere et al., 2001, 2003a, 2003b; van Die and Cummings, 2006). Recent data showed that vaccination with natural excretory/secretory (ES) antigens from *H. contortus* in Alhydrogel, a strong Th2 type response-inducing adjuvant, induced protection in lambs against challenge infection with *H. contortus*, whereas a similar vaccination protocol using dimethyl dioctadecyl ammonium bromide (DDA) as adjuvant was ineffective (Vervelde et al., 2003). In these vaccination trials, induction of protection was significantly correlated with the presence of high levels of serum IgG against the glycan epitope GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF), suggesting that this glycan structure may contribute to the induction of protective immunity (Vervelde et al., 2003).

Novel developments in glycan microarray technology now allow the simultaneous detection of antibodies directed against a large number of glycan antigens using very small serum samples (Blixt et al., 2004). To explore whether vaccination with *H. contortus* ES antigens induces multiple anti-glycan antibodies, the same sera as used in our previous studies were screened on a glycan-array containing more than 250 different glycan antigens. The data indicate that vaccination of lambs with ES antigens indeed resulted in eliciting multiple anti-glycan antibodies, which varied depending on the adjuvant used. In addition to anti-LDNF

IgG, a high level of IgG recognizing the glycan antigen Galα1-3GalNAc was observed only in sera of the protected lambs, which were vaccinated with ES antigens in Alhydrogel. Our data revealed that glycoproteins from different developmental stages of *H. contortus* contain a terminal Galα1-3GalNAc-R moiety, a glycan antigen that to our knowledge has not been reported before on helminth glycoproteins.

Materials and methods

Materials

Sera from lambs were obtained from studies described previously (Vervelde et al., 2003). Essentially, Black Bless sheep were immunized s.c. three times at 3 week intervals (at day 0, day 21 and day 42) with *H. contortus*-derived ES products in Alhydrogel or DDA. Two weeks after the last immunization (day 56), all sheep were challenged with *H. contortus* L3s. *H. contortus* ES antigens were obtained as previously described (Vervelde et al., 2003). The lectin GSI-B4-biotin was purchased from Sigma (St. Louis, MO, USA). Goat anti-mouse-peroxidase (PO), streptavidin-PO and streptavidin-alkalic phosphatase were purchased from Jackson Immunoresearch (West Grove, USA). The anti-mouse-alkalic phosphatase was purchased from Zymed laboratories, Inc. (San Francisco, USA) and mouse anti-sheep IgG was from Serotec (Kidlington, UK). The anti-Galα1-3Gal antibody M86 (Galili et al., 1998) was a kind gift from Dr. U. Galili (University of Massachusetts Medical School, USA). Monocytes were isolated from buffycoat (Sanquin, Amsterdam, the Netherlands) with CD14 MACS beads (Miltenyi biotec, Auburn, USA) according to the manufacturer's protocol. Galα1-3Gal-polyacrylamide (PAA), Galα1-3GalNAc-PAA and glucitol-PAA were purchased from Lectinity (~20% substitution, Lectinity, Finland) and LDNF-BSA was synthesized as previously described (van Remoortere et al., 2000). p-Nitrophenyl-N-acetyl-β-D-GalNAc (GalNAcβ-pNP), GalNAcα-pNP, Galβ-pNP, Galα-pNP, Galβ1-4GlcNAcβ-pNP (LN-pNP) were purchased from Sigma (St. Louis, MO, USA). GalNAcβ1-4GlcNAc-O-$(CH_2)_8COOCH_3$ was a kind gift from Ole Hindsgaul (University of Alberta, Canada). Fucα1-2Galβ1-3GlcNAc-O$(CH_2)_7CH_3$, Fucα1-2Galβ1-4GlcNAc-O-$(CH_2)_8COOCH_3$ and Galβ1-3GlcNAc-O-$(CH_2)_8COOCH_3$ were a kind gift from Monica Palcic (University of Alberta, Canada).

Glycan array

Glycan array screening was performed by Core H of the Consortium for Functional Glycomics (CFG) (University of Oklahoma, Oklahoma, USA). The glycan array is a microarray containing a library of natural and synthetic glycans with amino linkers printed onto *N*-hydroxysuccinimide (NHS)-derivatized glass slides to form a covalent amide linkage. All glycan structures used and their CFG numbers (#), as well as standard procedures for glycan array testing are available at the CFG website (http://www.functionalglycomics.org/fg/). The array used was printed array Version 2.1 containing glycan structures with CFG # 1-264.

Glycan-array slides were incubated with pooled serum (day 49, 1:100 dilution), and subsequently

with Alexa-labeled mouse anti-sheep IgG secondary antibodies in PBS containing 0.5% Tween-20. The samples (100 µl) were applied directly onto the surface of a single slide, covered with a microscope cover slip and then incubated in a humidified chamber for 60 min. Slides were subsequently washed by successive rinses in (i) PBS-0.05% Tween, (ii) PBS, (iii) deionized water, and immediately subjected to imaging. Fluorescence intensities were detected by using a ScanArray 5000 (PerkinElmer) confocal scanner. Image analyses were carried out using IMAGENE image analysis software (BioDiscovery, El Segundo, CA, USA). No background subtractions were performed. The array was done twice. Data were plotted by using Microsoft EXCEL software.

Preparation of helminth proteins

Helminth homogenates were prepared from *H. contortus* (adults and L3s), *Dictyocaulus viviparus* (adults and L3s), *Trichinella spiralis* (L3s and ES antigens), *Toxocara canis* (adults), *Caenorhabditis elegans* (adults), *Fasciola hepatica* (adults), *S. mansoni* (adults and cercariae) as described by De Bose-Boyd et al. (1998). For Western blotting, frozen worms were thawed and resuspended in 100 mM Tris-HCl, pH 8, containing protease inhibitors. For ELISA assays, the proteins of the helminth homogenates were precipitated by adding 4 vol. of (-20°C) acetone. Subsequently, the mixture was incubated for 1 h at –20°C, the protein pellet collected by centrifugation for 10 min at 13,000 *g* and re-suspended in ELISA coating buffer. For galactosyltransferase assays, *H. contortus* adult worms were homogenized in 50 mM Na cacodylate buffer, pH 7, on ice using five pulses of 10 s with a Polytron PT 1200 (Kinematic AG Littau, Switzerland). After sonification, Triton-X-100 was added to a final concentration of 1% and the mixture was incubated on ice for 30 min. The supernatant was collected after centrifugation for 10 min 11,000 *g* at 4°C, and the protein concentration was determined using the BCA protein Assay (Pierce).

Affinity purification of anti-Galα1-3GalNAc antibodies from serum of immunized lambs

To purify antibodies specific for Galα1-3GalNAc, 0.75 ml pooled serum (day 49) was used, derived from the lambs immunized with ES antigens in Alhydrogel after vaccination. The serum was incubated with Galα1-3GalNAc-PAA-Biotin (1 mg/ml) for 30 min at room temperature in PBS containing 0.1% SDS (BDH Laboratory Supplies, Poole, England). The formed immune complexes were subsequently captured with streptavidin-agarose beads by incubation at room temperature for 1 h on a roller bank. The beads were collected by centrifugation and washed with PBS containing 0.1% SDS. The bound antibody was eluted from the beads with 0.1 M glycine-HCl, pH 2.8, immediately neutralized with 1 mM Tris, pH 7.5, (Pierce, Rockford,USA), and assayed for Galα1-3GalNAc specificity by ELISA.

ELISA and Western blotting

Helminth extracts, glucitol-PAA, Galα1-3Gal-PAA, Galα1-3GalNAc-PAA, LDNF-BSA (10 µg/ml) and lysates of monocytes (10 µg/ml) were coated overnight on NUNC maxisorb plates

(Roskilde, Denmark). After blocking (60 min 37°C) with 1% ELISA-grade BSA (Fraction V, fatty acid free; Calbiochem, San Diego, USA) in TSM (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) and washing with TSM containing 0.1% Tween-20, glycan-specific antibodies or biotinylated GSI-B4 were added for 60 min at 37°C. For the ELISA with sheep-derived serum, bound antibodies were detected by incubation with mouse anti-sheep IgG (Serotec, UK), followed by detection with goat anti-mouse PO, both at 37°C for 60 min. In the case of incubation with biotinylated GSI-B4, unbound GSI-B4 was washed away with TSM and binding was detected with streptavidin-PO conjugate. The reaction was developed by TMB substrate and O.D. measured by spectrophotometry.

For Western blotting, the proteins (15 µg) within the helminth extracts were separated by SDS-PAGE (Mini-PROTEAN 3 System, BioRad, Hercules, USA) under reducing conditions on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked overnight in 1% BSA/PBS solution and probed for 1 h at room temperature with GSI-B4-biotin. After washing and incubation with streptavidin-alkaline phosphatase conjugate, bound lectin was detected using x-phosphate/5-bromo-4-chloro-3-inodylphosphate (Promega) and 4-nitrobluetetrazolium chloride (Promega, Leiden, the Netherlands).

Galactosyltransferase assays

Galactosyltransferase activity of *H. contortus* homogenates and bovine α1,3galactosyltransferase (Sigma, Saint Louis, Missouri, USA) were determined essentially as previously described (Joziasse et al., 1990). Enzyme assays, using *H. contortus* extract or bovine α1,3galactosyltransferase as the enzyme source, were done in a 25 µl reaction mixture containing 0.5 mM UDP-[^{14}C]-Gal (6 Ci/mol) (Amersham Biosciences, Buckinghamshire, UK), 20 mM $MnCl_2$, 4 mM ATP, 0.5% Triton X-100, 100 mM Na-cacodylate buffer, pH 7.2, and 1 mM acceptor substrate. Control assays lacking an acceptor were performed to correct for endogenous acceptors. After incubating the samples for 17 h at 37°C, the mixture was passed through SepPak C-18 cartridges (Palcic et al., 1994) (Waters Corporation, Massachusetts, USA). The UDP-[^{14}C]-galactose incorporation was measured by liquid scintillation (Packard TRI CARB, A Camberra Company, Ontario, Canada). The average enzymatic activity of two independent experiments was defined in nmol/ml/h.

Results

Presence of anti-glycan antibodies in sera of lambs immunized with ES antigens of *H. contortus*

We have previously reported that lambs are protected against the parasitic nematode *H. contortus* after vaccination with ES glycoproteins using Alhydrogel as an adjuvant. When DDA was used as an adjuvant, no protection was seen (Vervelde et al., 2003). In lambs vaccinated with ES antigens in Alhydrogel, but not in any other group, a significant increase was found in antibody levels against the LDNF antigen, and the anti-LDNF IgG response was significantly correlated with protection.

To determine whether the protected lambs have, in addition to serum antibodies to LDNF, antibodies against other glycan antigens, the same sera as used in our previous studies were screened for antibodies recognizing specific oligosaccharides within a large library of glycan antigens, using the glycan-array facility of the CFG (http://www.functionalglycomics.org) (Blixt et al., 2004). The data in Fig. 1 show that sera from lambs vaccinated with ES antigens in Alhydrogel and DDA contained antibodies that recognized multiple glycan antigens on

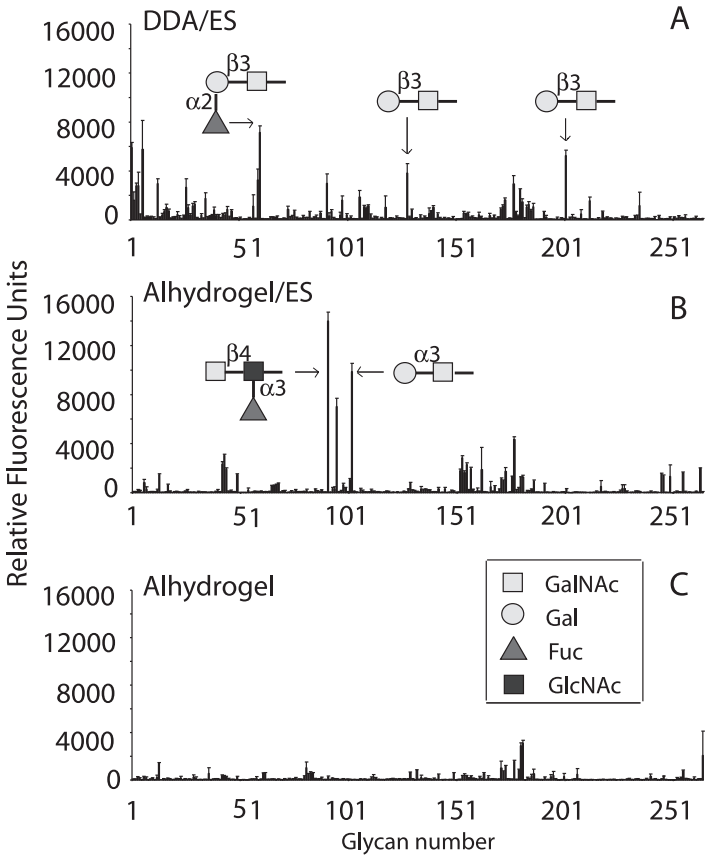

Figure 1. Glycan array analysis of anti-glycan antibodies in the sera of lambs immunized with *Haemonchus contortus* excretory/secretory (ES) antigens.

Pooled sera (1:100 diluted in PBS) from lambs immunized with *H. contortus* ES antigens in combination with adjuvant dimethyl dioctadecyl ammonium bromide (DDA) or Alhydrogel, contain IgG antibodies to different glycan antigens as determined by glycan array analysis. Pooled sera from lambs that received ES antigens in DDA contain mostly IgG antibodies recognizing Galβ1-3GalNAc, or α2-fucosylated Galβ1-3GalNAc (A), whereas sera from lambs that received ES antigens in Alhydrogel contain mostly serum IgG recognizing Galα1-3GalNAc and GalNAcβ1-4(Fucα1-3)GlcNAc-R (LDNF) (B).. Sera from lambs that received only adjuvant without ES antigens did not contain significant anti-glycan antibody levels (C).

the array, in contrast to pooled serum from a control group that only received Alhydrogel. Remarkably, the sera from the vaccinated lambs vaccinated with ES antigens and different adjuvants did not recognize the same glycan antigens. Similar to our previous observations, sera from lambs immunized with ES antigen in Alhydrogel recognized the LDNF antigen (#91, referring to the number assigned to this epitope in the array). In addition, the Galα1-3GalNAc glycan antigen (#102) was clearly recognized, as well as an oligosaccharide containing the blood group B-antigen (#95) which contains a terminal α1-3Gal. However, the related structure Galα1-3Galβ-R was not recognized by serum antibodies. In contrast, lambs vaccinated with ES antigens in DDA, a vaccination protocol that did not induce protection, contained serum antibodies recognizing both Galβ1-3GalNAc (#128, #201) and a fucosylated derivative, Fucα1-2Galβ1-3GalNAc (#59, #60).

To validate the data found in the glycan array, an ELISA was performed, in which neoglycoconjugates carrying different selected glycan antigens were coated. The amount of IgG-specific antibodies against Galα1-3GalNAc, LDNF and Galα1-3Gal in the sera of lambs immunized with ES antigens in combination with Alhydrogel or DDA, and the sera of control lambs immunized with Alhydrogel only, was measured. Similar to the results of the glycan array, high levels of IgG antibodies against both LDNF and Galα1-3GalNAc were detected only in the lambs immunized with ES antigens in Alhydrogel (Fig. 2), whereas no antibodies could be detected recognizing the Galα1-3Gal-epitope. The antibody levels observed were highest at day 49 of the immunization protocol, similar to what has been previously observed for the anti-LDNF antibody levels (Fig. 2).

Terminal α-Gal on glycoproteins from *H. contortus* and other helminths

The presence of antibodies recognizing the glycan antigen Galα1-3GalNAc in sera of lambs immunized with *H. contortus* ES antigens predicts that such glycans are synthesized within *H. contortus*. To establish the presence of terminal Galα1-3GalNAc-R moieties in glycoconjugates of *H. contortus*, antibodies recognizing Galα1-3GalNAc from the sera of lambs immunized with ES antigens in Alhydrogel were affinity purified by immunoprecipitation of the serum with Galα1-3GalNAc-PAA-biotin coupled to streptavidin beads. The antibodies eluted from the beads showed binding to Galα1-3GalNAc-PAA in ELISA as expected, whereas no binding to LDNF-BSA or Galα1-3Gal-PAA could be detected (Fig. 3A), thereby establishing that the antibody was highly purified. The immunopurified anti-Galα1-3GalNAc antibodies recognized glycoproteins in both *H. contortus* adults and ES antigens, whereas a lower binding was observed to L3s (Fig. 3B). In parallel, the presence of Galα1-3Gal glycan epitopes, recently described to occur in the nematode *Parelaphostrongylus tenuis* (Duffy et al., 2006), was investigated within *H. contortus* using the anti-Galα1-3Gal monoclonal antibody (mAb) M86 that does not recognize Galα1-3GalNAc epitopes (Galili et al., 1998) (Fig. 3A). The results show that the anti-Galα1-3Gal mAb M86 recognizes *H. contortus* ES glycoproteins, indicating the presence of terminal Galα1-3Gal glycan epitopes (Fig. 3B).

To investigate whether other helminth species contain glycan antigens terminating in α-Gal,

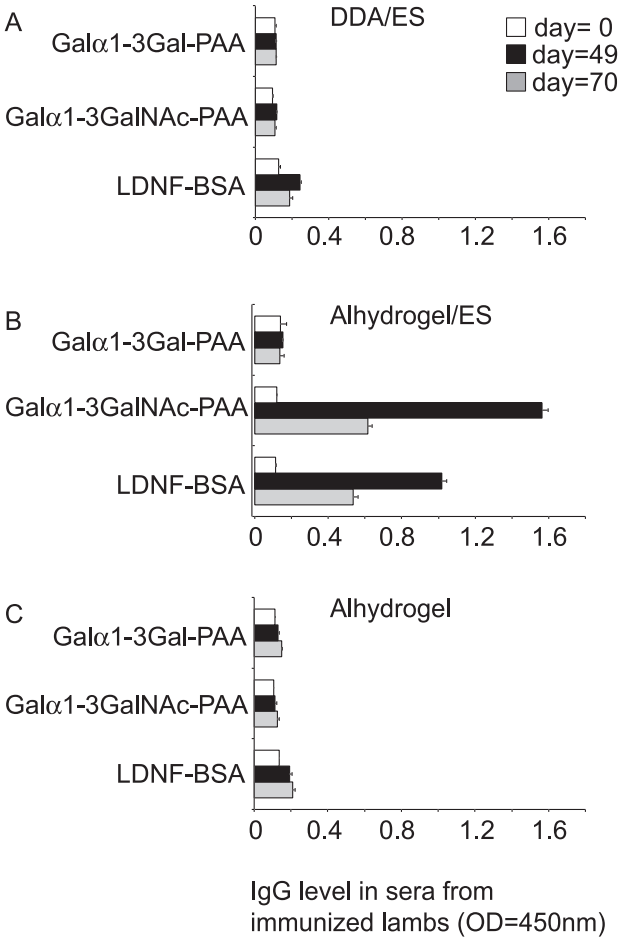

Figure 2. Anti-glycan IgG in the sera of lambs vaccinated with *Haemonchus contortus* excretory/secretory (ES) products analyzed by ELISA.

The amount of IgG against Galα1-3GalNAc-polyacrylamide (PAA), GalNAcβ1-4(Fucα1-3)GlcNAc-BSA (LDNF) and Galα1-3Gal-PAA, was determined by ELISA in pooled sera (1:100 diluted in PBS) of lambs immunized with ES antigens in dimethyl dioctadecyl ammonium bromide (DDA) (A) or ES antigens in Alhydrogel (B) or with Alhydrogel only (C), on different days in the immunization schedule (Vervelde et al., 2003). The data from two independent experiments, performed in duplicate, are shown and error bars represent the S.D

homogenates of different nematode and trematode species were tested with the lectin GSI-B4 by immunoblot and ELISA. GSI-B4 is a lectin showing a high specificity for α-Gal, whereas β-Gal is hardly bound (Murphy and Goldstein, 1977) (Fig. 4). GSI-B4 binds to glycoproteins from different life stages of *H. contortus* (Fig. 5A and B), and binding of GSI-B4 was observed to *T. canis* glycoproteins (Fig. 5A-C). The binding of GSI-B4 to *H. contortus* ES antigens and *T. canis* glycoproteins is specific, since it could be inhibited by raffinose but not by mannose (Fig. 5C). A very low or no detectable binding of GSI-B4 was observed to glycoproteins derived from *D. viviparous, T. spiralis, F. hepatica* and *S. mansoni,* or to a control glycoprotein mixture derived from human monocytes (Fig. 5A), indicating that terminal α-Gal is not a very common feature on glycoproteins of nematodes or trematodes. *Toxocara canis* adult worms did not show reactivity with the anti-Galα1-3Gal mAb (Fig. 5B), which may indicate that the α-Gal within *T. canis* glycoproteins is not present in an α1-3-linkage to Gal, but the exact structural details should be further investigated. In summary, our data provide evidence

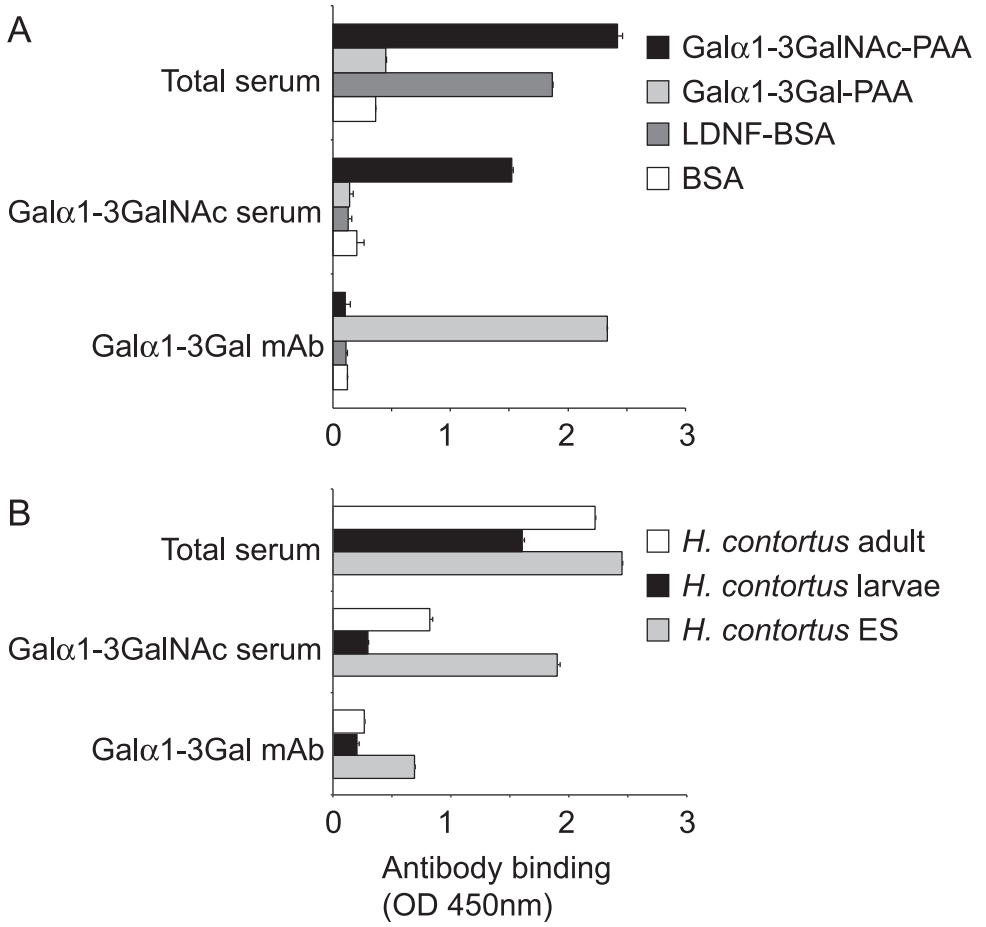

Figure 3. Glycoproteins of *Haemonchus contortus* contain Galα1-3GalNAc as well as Galα1-3Gal antigens.

A) Anti-Galα1-3GalNAc antibodies (indicated as Galα1-3GalNAc serum) were affinity purified from total serum of protected lambs (indicated as total serum), as described in Materials and methods. The Galα1-3GalNAc antibodies specifically recognize Galα1-3GalNAc- polyacrylamide (PAA), and not Galα1-3Gal-PAA or GalNAcβ1-4(Fucα1-3)GlcNAc-R (LDNF)-BSA, as was demonstrated by ELISA with these neoglycoconjugates (coated at 5 µg/ml). By contrast, the monoclonal antibody (mAb) M24 specifically detects Galα1-3Gal-PAA, which is in agreement with the reported Galα1-3Gal specificity of this antibody (Galili et al., 1998). **B)** The anti-Galα1-3GalNAc antibodies recognize adult worm proteins (coated at 10 µg/ml) and excretory/secretory (ES) glycoproteins (coated at 2 µg/ml) of *H. contortus*, whereas lower recognition of L3s (coated at 10 µg/ml) was detected, as shown by ELISA. mAb M24 (anti-Galα1-3Gal) shows binding to *H. contortus* ES glycoproteins, whereas binding to the other stages was hardly detectable.

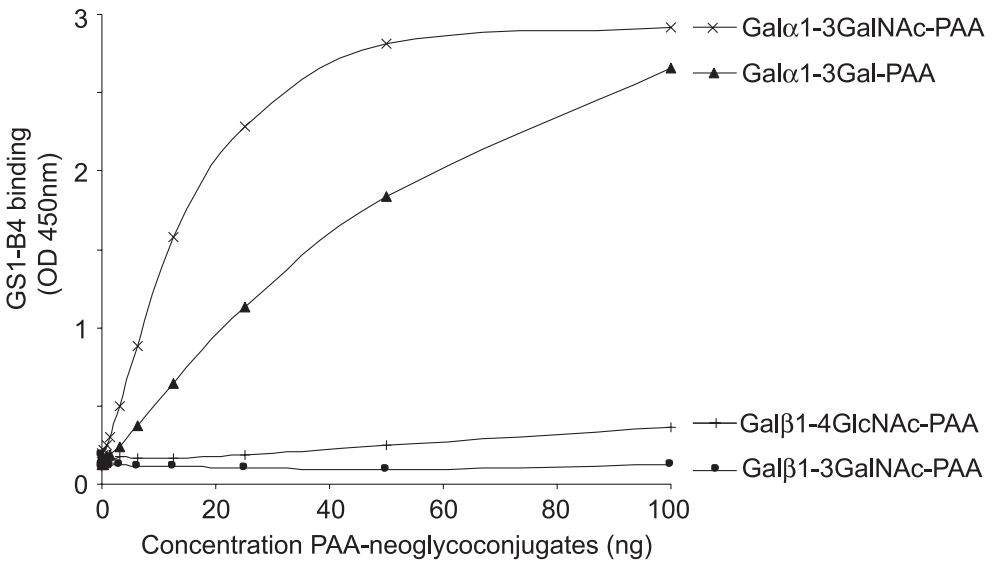

Figure 4. The lectin GSI-B4 recognizes neoglycoconjugates with terminal αGal and shows higher affinity to Galα1-3GalNAc-PAA than to Galα1-3Gal-PAA.
Neoglycoconjugates carrying different glycan antigens coupled to Polyacrylamide (PAA) were coated in similar concentrations ranging from 0 – 100 ng (0 – 2 μg/ml in coating buffer) and analysed for reaction with GSI-B4-PO (5 μg/ml) in ELISA.

that *H. contortus* expresses both Galα1-3Gal and Galα1-3GalNAc containing glycoproteins. Remarkably, glycoproteins containing terminal α-Gal epitopes are not frequently detected in other nematodes or trematodes, suggesting certain species-specificity.

Extracts of *H. contortus* contain galactosyltransferase(s) acting on oligosaccharides with a terminal GalNAc

To determine whether *H. contortus* expresses an enzyme capable of catalyzing the transfer of a Gal from UDP-Gal to substrates with terminal GalNAc, a homogenate of *H. contortus* adult worms was used as an enzyme source. The acceptor specificity of the putative *H. contortus* galactosyltransferase(s) compared with that of bovine α3-galactosyltransferase is shown in Table 1. Results indicate that *H. contortus* expresses galactosyltransferase(s) with activity towards several substrates with a terminal GalNAc, whereas no activity was detected towards any acceptor tested with a terminal Gal. The products formed by this enzyme activity, Gal-GalNAc-R, could not be cleaved by either α- or β-galactosidase (Sigma; data not shown), even when added in 100-fold excess, preventing the determination of the type of anomeric linkage of the Gal in the formed products. The bovine α1,3GalT, which was tested in parallel as a control, has a clear preference for mono- or oligosaccharides with terminal β-linked Gal (Table 1) as previously demonstrated (Joziasse et al., 1990). In conclusion, *H. contortus*

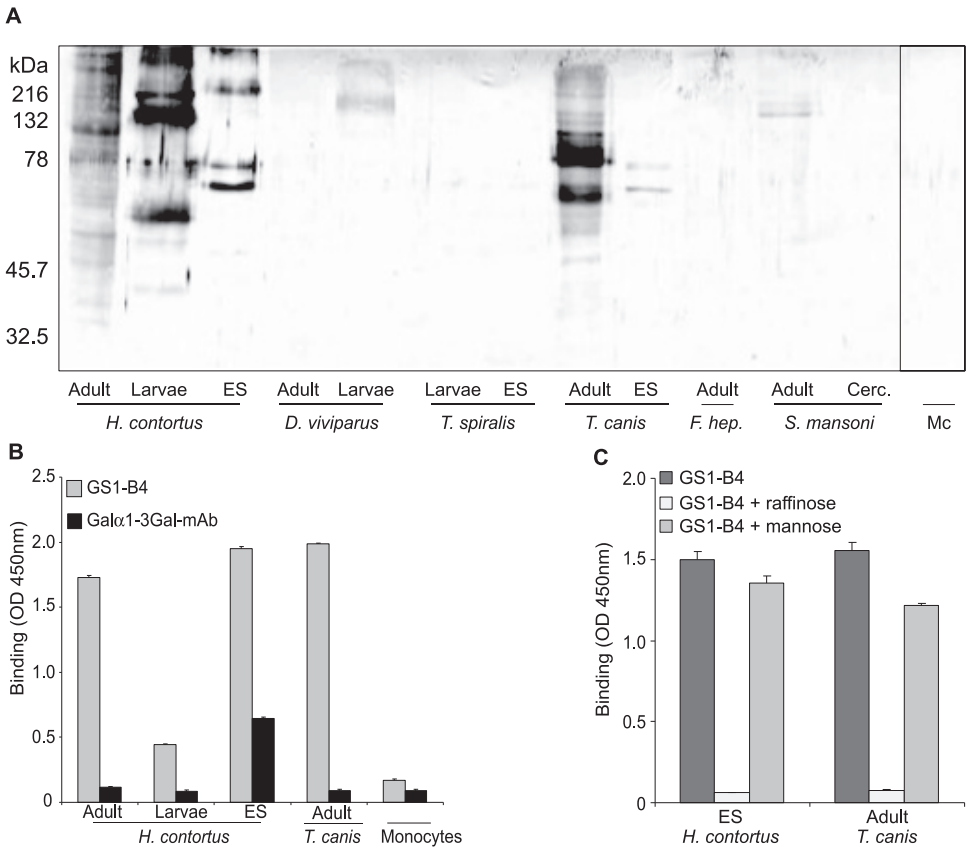

Figure 5. Glycoproteins from *Haemonchus contortus* and *Toxocara canis* react with the αGal-specific lectin GSI-B4.

A) Proteins (15 µg) of different helminth species and stages (indicated as in the figure, *Dictyocaulus viviparus, Trichinella spiralis, Schistosoma mansoni, Fasciola hepatica (=F. hep)*) were separated by SDS-PAGE and transferred to nitrocellulose. Monocyte-derived proteins (Mc) were included in the assay as a negative control. Western blots were incubated with the αGal-specific lectin GSI-B4 (biotin-labeled, 5 µg/ml). Molecular size markers (in kDa) are indicated to the left. B) *H. contortus* and *T. canis* proteins strongly react with GSI-B4, as was demonstrated by ELISA using GSI-B4-PO at 5 µg/ml. Analysis of the binding of the Galα1-3Gal-specific monoclonal antibody (mAb) M24 (Galili et al., 1998) showed that *H. contortus* excretory/secretory (ES) proteins, but not *T. canis* worm glycoproteins, contain detectable levels of terminal Galα1-3Gal. C) The staining of *H. contortus* and *T. canis* proteins with GSI-B4-PO (5 µg/ml) is specific, as was shown by the capacity of raffinose (10 mM), but not mannose (10 mM), to block the binding.

Table 1: Galactosyltransferase activity in an enzyme extract derived from *Haemonchus contortus* adult worms.

Acceptor	Relative galactosyltransferase (GalT) activity	
	H. contortus GalT	Bovine α1,3-GalT
GalNAcα-O-pNP	100[a]	<1
GalNAcβ-O-pNP	13	<1
GalNAcβ1-4GlcNAc-R_1 [#]	40	5
Galβ1-4GlcNAc-pNP	<1	51
Galβ-O-*p*NP	<1	41
Galα-O-*p*NP	<1	4
Fucα1-2Galβ1-3GlcNAc-R_2	<1	1
Fucα1-2Galβ1-4GlcNAc-R_1	<1	2
Galβ1-3GlcNAc-R_1	<1	100[a]

All acceptor substrates in the assays have been used at a concentration of 1 mM. The acceptor specificity of the *H. contortus* enzyme extract has been compared to the activity of commercial bovine α1,3-galactosyltransferase (α1,3-GalT). For both enzymes, the acceptor substrate that showed the highest activity has been set at 100%. In the assays with *H. contortus* extract, 100% activity represents an enzyme activity of 1 nmol/ml/h, and for the bovine α1,3-GalT 100% activity represents an activity of 49 nmol/ml/h.
[a]Values set at 100%; [#]R_1= -O-$(CH_2)_8COOCH_3$; R_2= -$(CH_2)_7CH_3$

contains galactosyltransferase activities that are clearly distinct from bovine α1,3GalT. These galactosyltransferases may be responsible for the synthesis of both Galα-GalNAc and/or Galβ-GalNAc sequences in *H. contortus*.

Discussion

Lambs can be protected against challenge infection with the parasitic nematode *H. contortus* by vaccination with ES glycoproteins using Alhydrogel as an adjuvant (Vervelde et al., 2003). In the studies described, a high IgG antibody level against LDNF was observed in sera of the animals vaccinated with ES antigens in Alhydrogel, which was significantly correlated with protection (Vervelde et al., 2003). Here, we extend these findings by showing that the sera of the protected lambs also contained a high level of IgG antibodies against the glycan epitope Galα1-3GalNAc, as shown by glycan micro-array screening and confirmed by ELISA. Vaccination of lambs with ES antigens in DDA, which was not associated with protection, also showed induction of anti-glycan antibodies. Remarkably, these anti-glycan antibodies were directed to other glycan antigens than seen in the lambs vaccinated with ES antigens in Alhydrogel

The presence of serum antibodies against Galα1-3GalNAc antigens in the immunized animals indicates that *H. contortus* ES antigens contain these glycan moieties, and that these glycans are antigenic. The data show, to our knowledge for the first time, that Galα1-3GalNAc epitopes are present in H. contortus ES antigens and on glycoproteins of adult worms. Within glycolipids, Galα1-3GalNAc epitopes have been shown as a conserved structural motif within the arthro-series carbohydrate backbone of glycolipids in *C. elegans* (Gerdt et al., 1999), *Onchocerca volvulus* (Wuhrer et al., 2000) and *Ascaris suum* (Friedl et al., 2003), however it is unknown whether these glycolipids are immunogenic.

Screening of several helminth species with the lectin GSI-B4, which recognizes terminal α-Gal irrespective of its linkage, showed that this modification is not very common among nematodes and trematodes. In addition to *H. contortus*, a significant binding of GSI-B4 was detected with *T. canis*, whereas no or very low binding was observed with *S. mansoni*, *F. hepatica*, *T. spiralis* or *D. viviparus* glycoproteins. The presence of α-Gal as a capping structure of protein-linked glycans has been previously observed in some helminth species. In the dog cestode *Echinococcus granulosus*, N-glycans carry antennae capped with Galα-Gal (Khoo et al., 1997). In the nematode *Parastrongylus tenuis* which commonly infects white-tailed deer, Galα1-3Galβ1-4GlcNAc is present as a dominant antenna of complex type N-glycans in adult worms. Since deer, similarly to most non-human mammals synthesize Galα1-3Gal, Duffy et al. (2006) suggested that the presence of similar terminal glycan moieties in the worm may represent a form of molecular mimicry that could enable the nematode to evade the immune response of the host (Galili et al., 1988; Damian, 1997). Our data show a similar situation in *H. contortus*, which infects sheep that most likely synthesize Galα1-3Gal epitopes. No antibodies recognizing Galα1-3Gal could be detected in the sera of lambs immunized with *H. contortus* ES antigens, whereas our data (Fig. 3B) indicate that *H. contortus* ES glycoproteins express terminal Galα1-3Gal and Galα1-3GalNAc moieties.

The use of different adjuvants resulted in the induction of selective anti-glycan antibodies. The significance of this observation in relation to the induction of protection of the lambs to challenge infection is not fully clear. However, the induction of significant antibody levels to the glycan epitopes Galβ1-3GalNAc and Fucα1-2Galβ1-3GalNAc in lambs vaccinated with ES antigens in DDA might at least predict the presence of such glycan antigens in *H. contortus* and their antigenicity in sheep. Interestingly, a *C. elegans* α1,2-fucosyltransferase was recently characterized with the potential to generate the sequence Fucα1-2Galβ1-3GalNAcα-R, and various highly antigenic methylated forms of the Fucα1-2Galβ1-3GalNAc moiety have been demonstrated in *T. canis* (Khoo et al., 1991; Schabussova et al., 2007). These data suggest that Fucα1-2Galβ1-3GalNAcα-R may be a common structure in *H. contortus*, *C. elegans* and *T. canis*.

The presence of various terminal Gal residues in *H. contortus* implies that this nematode expresses enzymes capable of catalyzing the transfer of a Gal residue to oligosaccharides with a terminal GalNAc. Our results demonstrate that *H. contortus* adults indeed possess such galactosyltransferase activity. The potential of galactosyltransferase(s) from *H. contortus* to use acceptors containing a terminal GalNAc residue clearly differs from the preference of the bovine α1,3-galactosyltransferase, which only shows an efficient activity towards acceptors containing a terminal Gal (Joziasse et al., 1990). Unfortunately, the nature of the glycosidic bond between the Gal and GalNAc residues could not be determined using α-, or β-galactosidases. This could be the result of a reduced accessibility of the enzymes to the formed product, which is an uncommon substrate for the α- and β-galactosidases. The lack of activity towards H-type antigens may indicate that *H. contortus* does not express galactosyltransferases able to synthesize blood group B antigen, which contains a Galα1-3Gal moiety.

Our data illustrate that glycan microarrays constitute a promising technology for fast and specific analysis of serum anti-glycan antibodies in vaccination studies. In addition, this approach facilitates the discovery of novel, antigenic parasite glycan antigens that may have potential for developing glycoconjugate vaccines or utilization in diagnostics. It should be emphasized, however, that the glycan-array used in this study contains many (mostly known) glycan structures, but a great variety of glycan structures exist that are not present on the array. Very few *H. contortus* glycan structures have been structurally characterized (Haslam et al., 1996, 1998). It is expected that these nematodes express a large array of different glycan structures, possibly including highly antigenic structures, which are not present on available glycan-arrays. The availability of specific "pathogen-arrays" would allow enormous progress in this field.

In summary, the glycan-array screening reported here resulted in the discovery of the antigenic glycan structure Galα1-3GalNAc on glycoproteins of *H. contortus*, which has not been previously reported. The production of antibodies against this structure by sheep, protected by immunization with ES antigens in combination with Alhydrogel, suggests that these antibodies may contribute to immune protection, which is an interesting possibility warranting follow-up studies. An example illustrating the protective capacity of anti-glycan antibodies is the rapid expulsion of *T. spiralis* from rats by anti-tyvelose mono- and polyclonal antibodies (Ellis et al., 1994). Interestingly, vaccination of sheep using a galactose-containing protein complex from *H. contortus*, H-Gal-GP, showed a protective effect (Smith et al., 1994, 1999). Similar to ES antigens, H-Gal-GP contains the LDNF antigen, next to undefined Gal moieties. Recently it was shown that the protective effect that was obtained with this H-Gal-GP is unlikely to be caused by the LDNF epitope present on the metalloprotease that is part of the protein complex (Geldhof et al., 2005). Unfortunately, the structure of the Gal-containing "H-Gal-GP" is not yet known. It would be interesting to investigate whether protective epitope(s) on the H-Gal-GP complex could include Galα1-3GalNAc antigen.

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CHAPTER 3

Chemo-enzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF

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Abstract

Several parasitic helminths such as the human parasite *Schistosoma mansoni,* express glycoconjugates that contain terminal GalNAcβ1-4(Fucα1-3)GlcNAcβ-R (LDNF) moieties. These LDNF glycans are dominant antigens of the parasite, and are recognized by human dendritic cells via the C-type lectin DC-SIGN. To study the functional role of the LDNF antigen in interaction with the immune system, we have developed an easy chemo-enzymatic method to synthesize multivalent neoglycoconjugates carrying defined amounts of LDNF antigens. An acceptor substrate providing a terminal N-acetylglucosamine was prepared by coupling a fluorescent hydrophobic aglycon, 2,6-diaminopyridine (DAP), to N,N'-diacetylchitobiose. By the subsequent action of recombinant *C. elegans* β1,4-N-acetylgalactosaminyltransferase and human α1,3-fucosyltransferase VI (FucT-VI) this substrate was converted to the LDNF antigen. We showed that human FucT-VI has a relatively high affinity for the unusual substrate GalNAcβ1-4GlcNAc (LDN) and this enzyme was used to produce micromolar amounts of LDNF-DAP. The synthesized LDNF-DAP was coupled to carrier protein via activation of the DAP-moiety by diethyl squarate. By varying the molar glycan:protein ratio, neoglycoconjugates were constructed with defined amounts of LDNF, as was determined by MALDI-TOF analysis and ELISA using an anti-LDNF-antibody.

Keywords: fucosyltransferase; neoglycoconjugate; LDNF; 2,6-Diaminopyridine; Diethyl squarate

Introduction

Glycan molecules linked to proteins or lipids play important roles in cellular communication, adhesion and signalling and are key molecules in regulation of immune responses. To establish the role of individual glycans in diverse aspects of biology, the availability of neoglycoconjugates that carry defined glycan antigens is of crucial importance. Neoglycoconjugates are attractive tools to define anti-glycan responses in infection or immunization,[1, 2] or to define specific carbohydrate recognition by lectin receptors that occur on many immune cells.[3] Neoglycoconjugates are also used in vaccines to elicit carbohydrate-specific antibodies that can confer protection to infection, for example to *Neisseria meningitides and Streptococcus pneumoniae*.[4, 5]

Helminth parasites express a variety of unusual glycan antigens that are highly antigenic in infection. In addition, recognition of helminth glycan antigens by C-type lectin receptors on dendritic cells may modulate dendritic cell function.[6, 7] Of particular interest is the LDNF structure, which has been demonstrated in multiple helminth species such as *Schistosoma mansoni*, *Haemonchus contortus* and *Dirofilaria immitis*.[1, 8, 9] High levels of anti-LDNF antibodies have been detected in humans and mice infected with the trematode *Schistosoma mansoni*.[10, 11] The LDNF structure has also been found capped with tyvelose on the nematode *Trichinella spiralis*.[12-14] Moreover, lambs vaccinated with excreted/secreted *H. contortus* antigens showed high serum levels of anti-LDNF IgG which correlated with protection against the parasite.[1] The C-type lectin DC-SIGN, a pathogen receptor on human dendritic cells, recognizes LDNF and monoclonal antibodies that recognize LDNF inhibited the binding of DC-SIGN to soluble egg antigens derived from *S. mansoni*.[7, 15] To study the roles of LDNF in antigenicity and immune modulation in more detail, we have developed a method to synthesize neoglycoconjugates carrying LDNF, which are not commercially available.

In a previous study we have synthesized the LDNF structure using partially purified glycosyltransferases from natural sources.[16] In this manuscript we describe a strongly improved chemo-enzymatic method using recombinant glycosyltransferases, starting with the chemical modification of a commercially available acceptor with 2,6-diaminopyridine (DAP).[17] This provided a fluorescent hydrophobic aglycon to the acceptor, which facilitated detection and purification of the oligosaccharide products during synthesis. Coupling of the glycans to protein was performed via diethyl squarate,[18] allowing the controlled addition of defined amounts of glycan antigens to any protein carrier, which enables the study of multivalency of glycan presentation and the importance of the nature of the carrier molecule.

Materials and methods

Reagentia

Acetonitrile, Ammonium formate, 2,6-diaminopyridine, GDP-fucose, UDP-GalNAc, N,N'-diacetylchitobiose, Galβ1,4GlcNAc-pNp, GlcNAc-pNP, $MnCl_2$, sodium cacodylate, Tris-HCl, NaCl, $CaCl_2$, $MgCl_2$, Tween 20, diethyl squarate (3,4-Diethoxy-3-cyclobutene-1,2-dione) were

all obtained from Sigma and the BSA (Albumin, Bovine Serum, Fraction V) used for producing neoglycoconjugates was obtained from Calbiochem. The manufacturers from other reagents that were used are mentioned in the appropriate sections.

Derivatization of chitobiose with 2,6-diaminopyridine (DAP)

N,N'-diacetylchitobiose (chitobiose) was derivatized with 2,6-diaminopyridine (DAP) using essentially the method described by Xia et al.[19] Dried pellets of 2 µmol (0.85 mg) chitobiose were dissolved in 20 µl DMSO and subsequently 1 ml of a mixture of 0.27 M DAP and 0.78 M $NaCNBH_3$ in DMSO:HAc (7:3) was added. After 16 hours of incubation at 65°C, toxic materials and free chitobiose were removed using reverse phase HPLC cartridges and the DAP-derivatized oligosaccharide (Chi-2-DAP) was subsequently separated from free DAP on a preparative Zorbax NH_2 PrepHT column (250 x 21.2 mm, 7 µm, Agilent) in batches of 4 µmol (2.1 mg) per run. For this purpose, a gradient was used that started with 81% acetonitrile (buffer B) and 29% 50 mM ammonium formate, pH 4.4 (buffer A) and ended after 17 minutes with 30% acetonitrile and 70% ammonium formate on an Äkta Explorer with a column flow of 10 ml. DAP-containing fractions were detected by measuring absorbance at 235 nm. The Chi-2-DAP was freeze-dried and stored in pellets of 1 µmol (0.5 mg).

Production of recombinant β1,4-N-acetylgalactosaminyltransferase.

For the production of recombinant *C. elegans* UDP-GalNAc:GlcNAc β1,4-*N*-acetylgalactosaminyltransferase (β1,4GalNAcT), HEK293T cells were cultured adherent in DMEM containing 10 mM HEPES, MEM non essential amino acids, MEM sodium pyruvate, 10% FCS (all reagents from Gibco) and 100 U/ml peniciline-streptavidine (Lonza), in a T75 flask (Greiner) at 37°C/5% CO_2 until the bottom was completely covered by a monolayer of cells (~$6*10^6$ cells/flask). Lipofectamin transfection of these cells with plasmid pCMV-SH-Ceβ4GalNAcT [20] was performed according to manufacturer's (Invitrogen) protocol with some slight modifications. The transfection mixture was prepared by mixing 600 µl Optimem I (Gibco) containing 54 µl Lipofectamin 2000 (Invitrogen) with 600 µL Optimem I containing 9 µg DNA, and subsequently incubated for 30 minutes at room temperature. After washing the cells twice with 9 ml Optimem I, 4.8 ml Optimem I was mixed with the transfection mixture and added to the cells. After incubation of the cells for 5-6 hours at 37°C/5% CO_2, 6 ml culture medium was added and the cells were cultured overnight at 37°C/5% CO_2. Hereafter, the cells were washed with 2 ml PBS, trypsinized with 1 ml Trypsin (Gibco)/EDTA (containing 0.025% trypsin and 0.01% EDTA), and then cultured in 9 ml of culture medium in a new T75 flask for another 3 days. The medium containing the active enzyme was harvested daily until 7 days after transfection. The enzyme-containing medium showed an activity of approximately 200 nmol/ml/h using acceptor GlcNAc-pNp and was stored until further use at -20°C for several months without loss of activity.

Production of recombinant human α1,3-fucosyltransferases

The human α1,3-fucosyltransferases FucT-III, FucT-IV, and FucT-VI were kindly provided by H. Kok (Organon, the Netherlands) and the expression plasmid pAMO-FucT-IX, [21] encoding cDNA from human FucT-IX, [22] was kindly provided by Dr. T. Sato & Dr. H. Narimatsu (RCG, Japan). The latter construct was transfected into HEK293T cells as described in the previous section. and the cell lysates were used as enzyme source.

The plasmid ProtA-FucT-VI,[23] encoding a soluble chimaeric protein consisting of part of Protein A fused to the catalytic domain of FucT-VI, was kindly provided by Dr. B. Macher (San Francisco State University). The transfection procedure of plasmid was as described in the previous section, with the exception that Cos7 cells were used. The ProtA-FucT-VI containing medium was harvested 2 days after transfection and stored until further use at -80°C for several months without any loss of activity (approximately 100 nmol/ml/h measured with acceptor LDN-C_8).

Fucosyltransferase assays and enzyme kinetics

Standard fucosyltransferase assays were performed at 37 °C for 1 hour in a total volume of 25 µl 50 mM sodium cacodylate, pH 7.0, containing 1 µmol $MnCl_2$, 2,5 nmol GDP-[^{14}C]Fuc (4 Ci/mol, Amersham), 0.1 µmol ATP, 0.1% Triton X-100, acceptor substrates and enzyme, essentially as described.[24] The acceptor substrates were all used at a concentration of 1 mM unless indicated otherwise. GalNAcβ1-4GlcNAcβ-O-$(CH_2)_8$-$COOCH_3$ (LDN-C_8), and Fucα1-2Galβ1-4GlcNAc-O-$(CH_2)_8$-$COOCH_3$ (H-type 2-C_8) were kind gifts from Dr. D.H. van den Eijnden (VU University, NL), and Dr. M.M. Palcic (Carlsberg Laboratory, Denmark), respectively. Galβ1-4GlcNAcβ-Nitrophenyl (LN-pNp) was from Toronto Chemicals. Products were separated from unincorporated nucleotide sugar by reverse-phase chromatography using Sep-Pak C_{18} cartridges (Waters, Milford, MA).[25] Kinetic characterization of human ProtA-FucT-VI was carried out using at least six different acceptor substrate concentrations. The apparent kinetic parameters V_{max} and K_m were obtained by fitting the data to the Michaelis-Menten equation using nonlinear regression analysis (SPSS 15 program, SPSS Inc.).

Preparative enzymatic synthesis of oligosaccharides.

Four micromoles (2.1 mg) of Chi-2-DAP were incubated in a volume of 400 µl 0.1 M sodium cacodylate pH 7.0, containing 40 mM $MnCl_2$, 10 mM UDP-GalNAc, and medium from HEK293T cells transfected with the recombinant β1,4-GalNAcT from *Caenorhabditis elegans* [20] as enzyme source. The mixture was incubated at room temperature (20-24°C) until Chi-2-DAP was completely converted to the desired end-product, LDN-DAP. The product was purified by Sep-Pak C_{18} reverse-phase chromatography.[25] For the fucosylation of LDN-DAP, batches of 1 µmol (0.72 mg) LDN-DAP were incubated each in a total volume of 200 µl 50 mM sodium cacodylate buffer, pH 7.0, containing 5 mM $MnCl_2$, 10 mM GDP-L-fucose and ProtA-FucT-VI as enzyme source. The mixture was incubated at 37°C until LDN-DAP was completely converted to the desired end-product, LDNF-DAP. The product was purified by Sep-Pak C_{18} reverse-phase chromatography.[25]

Analytical HPLC analysis

During enzymatic synthesis, product formation was monitored in time by HPLC on a Surveyor (Thermo), applying 100-500 pmol oligosaccharide in 80% acetonitrile onto a LudgerSep N1 Amide column (250 x 4.6 mm, Ludger). A 30-minute gradient starting with 70% acetonitrile and 30% 50 mM ammonium formate, pH 4.4 and ending in a 50:50 ratio was run at 22°C to separate the formed product from the starting oligosaccharide. The column was subsequently equilibrated with 100% ammonium formate for 10 minutes, followed by 10 minutes of 70% acetonitrile and 30% 50 mM ammonium formate. Detection by fluorescence (Waters fluorimeter 470) was done with excitation at 345 nm and measuring the emission at 400 nm.

Construction of neoglycoconjugates

Four µmol (3.5 mg) LDNF-DAP was dissolved completely in 1 ml of 0.1 M phosphate buffer pH 6.95 and transferred to a glass coned vial (Wheaton). A freshly made mixture of 1 ml ethanol (via Brunschwig Chemie from Nedalco) with 20 µl 3,4-Diethoxy-3-cyclobutene-1,2-dione, (98%, Sigma Aldrich) was added and mixed. The sample was incubated at 22°C for 16 h. The addition of squarate to the LDNF-DAP was checked by electrospray ionization mass spectrometry (ESI-MS) (0.5 µl of the 2 ml reaction mixture is added to 50 µl 50% Acetonitrile). Hereafter, 30 ml of Milli Q water was added to the reaction mixture that was subsequently applied to a 6 cc Sep-pak column for purification.

For the final conjugation step, typically 10 µl of a solution of 5 nmol squarate-derivatized LNDF-DAP/µl conjugation buffer (made by dissolving 1.55 g boric acid (Gibco) and 1.31 g KCl (Fluka) in 30 ml Milli Q water; pH adjusted to 9 with 421 mg KOH (Merck)) was added to 200 µg BSA (2 µl of 0.1 mg/ml conjugation buffer) and 3 µl conjugation buffer giving an end-volume of 15 µl. This particular reaction provides a molar ratio of 17 glycans to 1 carrier molecule, but also other molar ratios were tested, varying from 17:1 to 3:1, always with the same amount of BSA in an end-volume of 15 µl. The reaction mixture was incubated at 22°C for 23 h. To remove salts, 1.1 ml Milli Q water was added to the reaction mixture and the sample was injected in a Slide-A-Lyzer Dialysis Cassette (10.000 MWCO, Thermo Scientific) and dialyzed against 5 l demi-H_2O for at least 16 h at 4°C. The neoglycoconjugates were dried in the speedvac and stored at –20°C.

Treatment of LDNF-DAP with α1,3/4-fucosidase

To determine the anomeric linkage of the fucose in LDNF-DAP we treated 20 nmol of this compound with 0.5 mU of α1,3/4-fucosidase (from *Xanthomonas sp.*,Calbiochem) for 18 hours in 20 µl 50 mM NaH_2PO_4 buffer, pH 5.5. The results were monitored with HPLC as described in previously.

Mass spectrometry

Synthesized oligosaccharide products were characterized by ESI–MS on an LCQ DecaXP ion trap mass spectrometer equipped with a nano-ES ionization source (Thermo Finnigan). Sample

was loaded onto a medium NanoEs spray capillary (Proxeon). The capillary temperature was set to 200°C. Spectra were taken in the positive ion mode with a spray voltage of 1.0 kV and a capillary voltage of 40.1 V.

To estimate the degree of coupling of the oligosaccharides to BSA, 5-10 pmol of the neoglycoconjugates was taken up in 50% of a sinapinic acid solution (17.5 mg/ml sinapinic acid (Sigma) in 281 µl MeOH and 422 µl acetonitrile), applied to the target plate and subjected to MALDI-TOF, using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) by measuring in Linear Mode (m/z range was 30,000-100,000 Da) and accumulating 5000 laser shots/MS spectrum.

ELISA

To determine the presence of the LDNF-epitope on the BSA carrier, 5 µg protein/ml coating buffer (50 mM Na_2CO_3 (Merck), pH 9.6) was added to each well of a Nunc Maxisorb ELISA plate and incubated at 4°C for 16 h. After washing three times with TSM (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) containing 0.1% Tween 20, blocking solution (TSM containing 1% BSA) was added for 1 h at 37°C. After a similar washing procedure, an anti-LDNF monoclonal antibody (SmLDNF1)[11] (kindly provided by Dr. R.D. Cummings and A.K. Nyame, Emory University, Atlanta, USA) was added in a 1:500 dilution in blocking solution and incubated at 37°C for 1h. After washing again, a peroxidase-conjugate Goat anti-Mouse IgG/IgM (H+L) (Jackson) was added in a 1:2500 dilution in TSM/0.1% Tween-20 and incubated for 1 h at 37°C. The plate was developed with 3,3',5,5'-tetramethylbenzidine (Sigma) after a final washing step. The color reaction was stopped by the addition of 50 µl 0.8 M H_2SO_4 to each well and absorbance was measured at 450 nm.

Results

Derivatization of chitobiose with 2,6-diaminopyridine (DAP)

As the starting backbone for the synthesis of LDNF, N,N'-diacetylchitobiose (chitobiose, Figure 1) was used, providing the terminal GlcNAc required for elongation to LDN. Two micromoles of chitobiose were derivatized with 2,6-diaminopyridine (DAP) (Figure 1, reaction a) using essentially the method described by Xia et al.[19] During this procedure the GlcNAc residue at the reducing end of chitobiose looses its ring-structure (Figure 1). Such addition of a DAP moiety provides the oligosaccharide with a hydrophobic and fluorescent aglycon spacer which greatly facilitates purification and detection of the oligosaccharides in subsequent synthesis steps. The DAP-derivatized oligosaccharide was separated from free DAP by preparative HPLC and DAP-containing fractions were detected by UV (Figure 2). The fractions eluting from the column after 12 minutes were analyzed by mass spectrometry (MS) (data not shown) to confirm the addition of DAP to Chi-2 (Figure 2, Chi-2-DAP). The Chi-2-DAP was freeze-dried in pellets of approximately 1 µmol and used for subsequent enzymatic synthesis reactions.

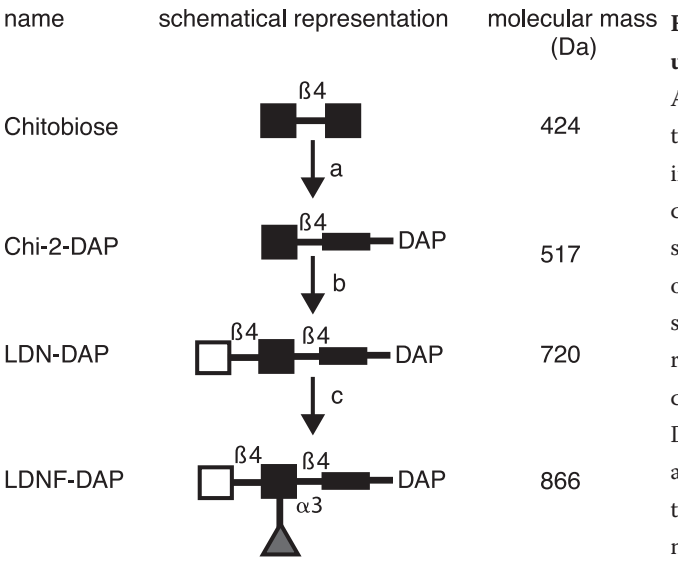


Figure 1. Oligosaccharides used in this study.

A schematic representation of the oligosaccharides mentioned in this paper and their molecular mass is depicted. Filled square (N-acetylglucosamine), open square (N-acetylgalactosamine), grey triangle (fucose), rectangle (derivatized, open-chain N-acetylglucosamine), DAP (2,6-diaminopyridine). In addition, the short names of these oligosaccharides and their molecular masses are given.

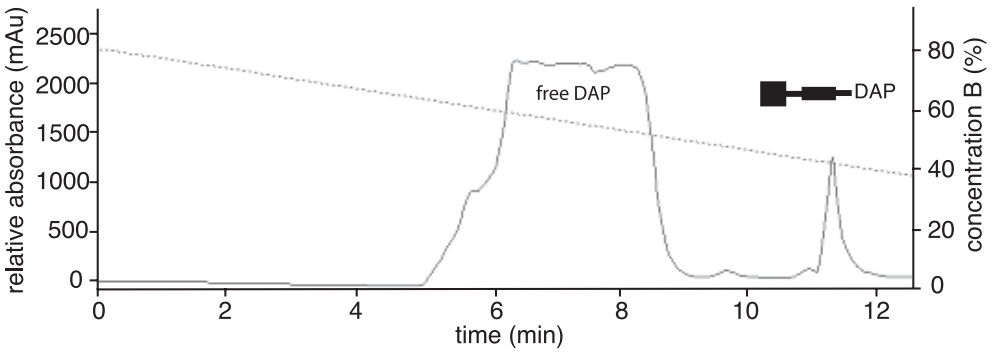


Figure 2. Purification of Chi-2-DAP.

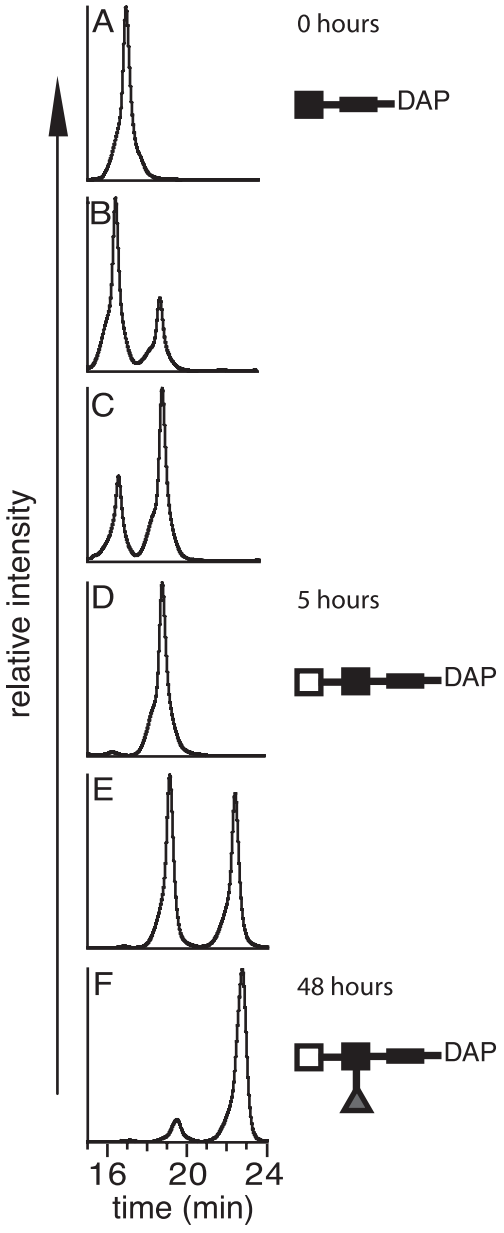
Chitobiose derivatized with DAP (Chi-2-DAP) was purified by reverse-phase chromatography to remove $NaCNBH_3$ and free chitobiose. DAP-derivatized chitobiose was separated from free DAP by preparative normal-phase HPLC. Relative absorbance of DAP was measured at a wavelength of 235 nm and fractions containing Chi-2-DAP were collected (peak at min 11.5). The dotted line shows the concentration of buffer B (acetonitrile) in the gradient used.

Enzymatic synthesis of LDNF-DAP

As a first step in the synthesis of LDNF-DAP, the produced Chi-2-DAP was converted to LDN-DAP (Figure 1) using the recombinant β1,4-GalNAcT from *Caenorhabditis elegans* [20] and UDP-GalNAc. Four micromoles of Chi-2-DAP were completely converted to the desired end-product, LDN-DAP, within 5 hours as monitored by analytical HPLC (Figure 3A-D). After purification of the product by C_{18} reverse phase chromatography, the structure of the

Figure 3. Product formation during enzymatic synthesis.

During enzymatic synthesis samples were analyzed by analytical HPLC. The relative intensity of DAP-derivatized glycans is plotted against time of elution from the column. In panel **A** Chi-2-DAP is shown, which is converted with β1,4-GalNAcT in time into LDN-DAP (**B-D**). After isolation of LDN-DAP with reverse-phase cartridges, this compound is in turn converted in time to LDNF-DAP with ProtA-FucT-VI (**E-F**).



oligosaccharide was verified to be LDN-DAP by tandem MS (data not shown). We previously demonstrated by NMR the authenticity of the product LDN generated by recombinant *C. elegans* β1,4GalNAcT. [20]

LDNF has been synthesized before using human milk containing different fucosyltransferases.[16] To improve the efficiency of this synthesis step, we evaluated the capacity of different recombinant human α1,3-fucosyltransferases to use LDN as an acceptor structure, using a

Table 1. Apparent kinetic parameters for recombinant FucT-VI[a]

Acceptor		Km (mM)	Vmax (nmol/mL/h)
Galβ1-4GlcNAc-pNpb	(LN-pNp)	3.6	41
GalNAcβ1-4GlcNAc-DAPc	(LDN-DAP)	3.8	50
GalNAcβ1-4GlcNAc-C8d	(LDN-C8)	0.27	120
Fuc-α1-2Galβ1-4GlcNAc-C8	(H-type 2-C8)	0.14	160
Galβ1-4GlcNAc-C8	(LN-C8)	0.22 / 0.31	Km estimated by 23, 24

[a]The apparent kinetic parameters K_m and V_{max} were obtained by fitting the data for the transfer of fucose to the acceptor substrates at variable acceptor concentrations (for LN-pNp: 0.1 - 5 mM, for LDN-DAP 0.05 – 2 mM, for LDN- C_8 0.05 – 1 mM, and for H-type2-C_8 25 µM – 150 µM) to the Michaelis-Menten equation using nonlinear regression analysis (SPSS 15 program, SPSS Inc.). The concentration of GDP-fucose (0.1 mM), and other assay conditions were similar, [b]pNp = p-Nitrophenyl, [c]DAP=2,6-diaminopyridine, [d]C_8 = $(CH_2)_8$-$COOCH_3$

standard radioactive fucosyltransferase assay. Among the fucosyltransferases tested (FucT-III, FucT-IV, FucT-VI and FucT-IX), FucT-VI showed the highest catalytic activity towards LDN-C_8 as acceptor substrate, compared to LN-pNp as an acceptor (results not shown). To define the enzymatic properties of FucT-VI on LDN as a substrate in more detail, we used the chimaeric enzyme ProtA-FucT-VI, which was produced as a soluble secreted enzyme. The K_m and V values of ProtA-FucT-VI for the substrates LDN-C_8, LDN-DAP, LN-pNp and H-type2-C_8 were estimated (Table 1). Interestingly, the K_m value calculated for LDN-C_8 is in the same range as for H-type 2-C_8 and the K_m measured previously for LN-C_8,[24, 26] indicating that the enzyme has a high affinity for LDN as a substrate. The K_m values of ProtA-FucT-VI for LDN-DAP or LN-pNP as acceptor appeared much higher, indicating that the type of hydrophobic spacer influences the affinity of the enzyme for the acceptor substrate.

Starting with 1 µmol LDN-DAP as an acceptor and ProtA-FucT-VI as enzyme source, 90-95% conversion of LDN-DAP to LDNF-DAP was reached after incubation of 48 hours as monitored by HPLC (Figure 3D-F). The product was purified by C_{18} reverse phase chromatography followed by preparative HPLC, and the structure of the oligosaccharide was verified by tandem MS (Figure 4A). Whereas FucT-VI is known to add Fuc in an α1,3-linkage to Galβ1-4GlcNAc as acceptor, its action on LDN has not been verified previously. To establish the anomeric linkage of the Fuc in LDNF-DAP, we treated a sample of the synthesized putative LDNF-DAP with α1,3/4-fucosidase, and analyzed the product by HPLC. The data in Figure 4B show that the product obtained after fucosidase treatment shows a similar retention time as LDN-DAP (see Fig. 3), thereby confirming the authenticity of the synthesized LDNF-DAP.

Construction of BSA-LDNF neoglycoconjugates via activation with diethyl squarate

To couple LDNF-DAP to a protein carrier, we first added a diethyl squarate molecule to the free amino-group of the DAP moiety (Figure 5A). We combined the strategies described by Lefeber,[27] and Hou,[18] and using ESI-MS, we confirmed the formation of the desired molecule with an expected molecular mass of 990 Da (Figure 5C). No free LDNF-DAP was detected, indicating that squarate was coupled to LDNF-DAP with high efficiency.

After purification of the squarate-modified LDNF-DAP using C_{18} reverse phase chromato-

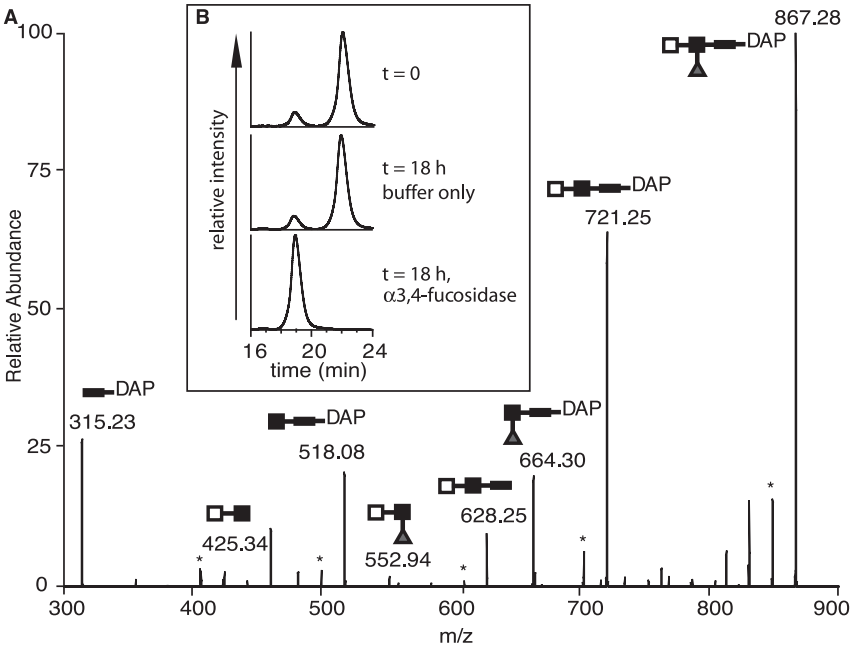


Figure 4. Characterization of LDNF-DAP by tandem mass spectrometry and α1,3/4-fucosidase treatment.

A.) Oligosaccharide products were characterized in positive mode by ESI-MS. The expected molecular mass of the protonated LDNF-DAP ion (867.28) was found, and by fragmentation using tandem MS the structure was confirmed. Schematic figures of the oligosaccharides representing the found fragments are depicted above the peaks. The asterisks above detected peaks indicate ions that are similar to the structures shown, but that lack a water molecule (minus 18 Da). B.) (insert) To characterize the anomeric linkage of the fucose, we treated LDNF-DAP in an acidic phosphate buffer without (middle panel) or with α1,3/4-fucosidase (lower panel) for 18 hours, as described in Materials and methods, and subsequently subjected the mixture to HPLC analysis. The upper panel (t = 0) shows the starting material before fucosidase treatment.

graphy, the oligosaccharides were coupled to BSA (Figure 5B) essentially as described.[18] Different ratio's of glycan to protein were used to create different numbers of glycan per carrier molecule. In this case, we used either a 17 times (high) or a 3 times (low) molar excess of oligosaccharide to BSA. The degree of coupling of the oligosaccharides to BSA was determined by MALDI-TOF analysis (Figure 6B-C). This revealed for the high coupling ratio an average peak-shift of around 7.3 kDa, implicating an average of 7.4 LDNF-epitopes per BSA molecule. For the reaction with a low coupling ratio, the average peak-shift was around 1 kDa, indicating that the BSA molecules contained on average one LDNF epitope. To investigate whether the LDNF moieties were intact, the binding of an anti-LDNF-antibody to the BSA-LDNF neoglycoconjugates was evaluated by ELISA. The data showed a strong binding of the antibody to both BSA-LDNF

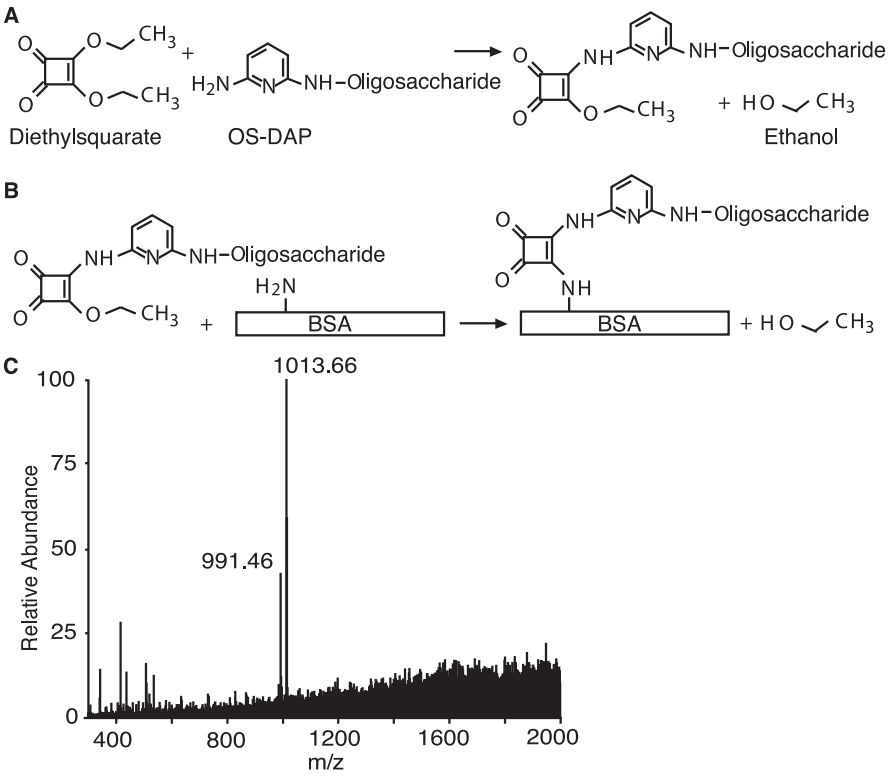


Figure 5. Diethyl squarate coupling and analysis.

Chemical reaction schemes for the coupling of diethyl squarate to DAP-derivatized oligosaccharides (A.) and the subsequent coupling of the protein carrier BSA (B.). ESI-MS analysis of the product after derivatization with diethyl squarate of LDNF-DAP is shown in (C.). Both the protonated (m/z of 991.46) and sodiated molecular ion (M/z of 1013.66) was measured.

preparations (Figure 6D, showing the antibody binding to BSA with the low LDNF content), but not to BSA (Figure 6D), confirming the structural integrity of the LDNF moiety.

Discussion

Ongoing research efforts in many labs aim at the development of improved methods to synthesize bio-active oligosaccharides to study, modulate, or inhibit processes that are dependent on interactions with carbohydrates. We have described here an easy method to synthesize milligrams of neoglycoconjugates, containing the LDNF-epitope, by a combination and optimization of different existing methodologies. The advantages of the use of DAP to derivatize oligosaccharides has been outlined before by Xia et al.,[19] The linkage of DAP to acceptor substrates destined for enzymatic synthesis has not been described before, and appears to have great advantages above other linker-adaptors. The use of DAP provides a

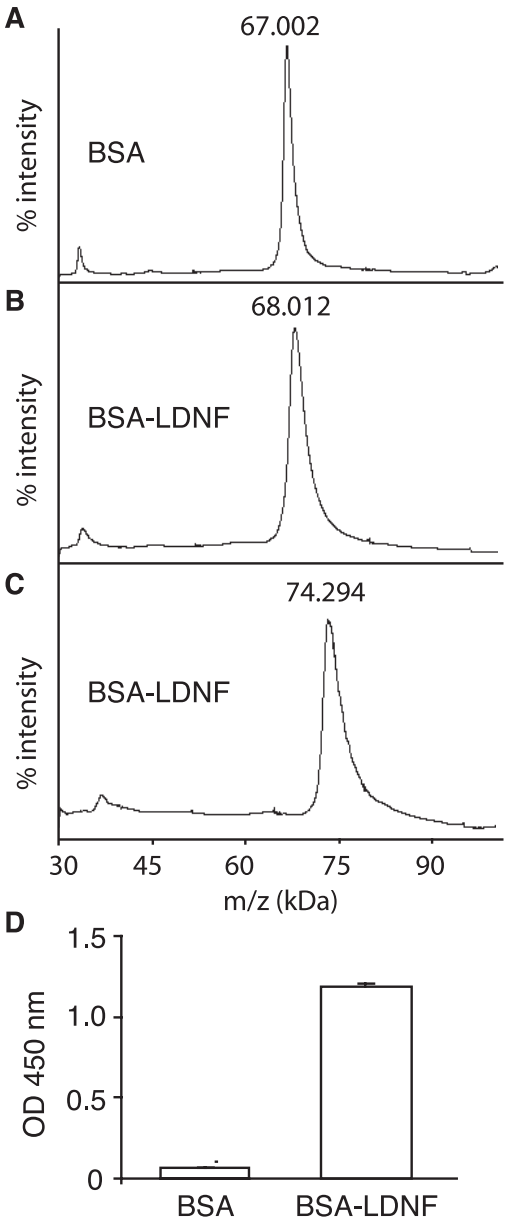


Figure 6. Analysis of BSA-LDNF with MALDI-TOF-MS and ELISA.

BSA-LDNF neoglycoconjugates that were produced with different molar ratios of sugar to the carrier molecule BSA, were analyzed by MALDI-TOF-MS and the molecular masses (B., C.) compared to those of untreated BSA (A.). LDNF-epitopes were detected on the BSA-LDNF with low ligand density using an anti-LDNF antibody in ELISA (D.). Untreated BSA was used as a negative control.

hydrophobic aglycon to the acceptor, allowing easy purification of the product after enzymatic reactions, and its fluorescent properties facilitate sensitive detection during the synthesis. In principle, each acceptor structure can be easily derivatized with DAP, facilitating enzymatic synthesis prior to coupling to carrier molecules without the need of specific spacer-linked acceptor substrates.

The study of the biological properties of particular helminth glycan antigens highly depends

on the availability of neoglycoconjugates, which in contrast to many mammalian-type glycans are not commercially available. Organic synthesis has been used successfully for the synthesis of typical helminth glycan antigens, as has been reported for example for fuco-oligosaccharides of *Schistosoma* spp,[28] or the mono- or dimethylated glycan antigens from *Toxocara canis.*[29] Alternatively, enzymatic approaches may be used, using glycosyltransferases, which offers significant advantages as it is fast and combines a high regio- and stereospecificity with the potential availability of many different glycosidic linkages. A drawback for the enzymatic synthesis of unusual glycans such as helminth glycans is that not many recombinant parasite-type glycosyltransferases are available. Previously, we have synthesized LDNF antigen using β1,4GalNAcT from the albumen gland of the snail *Lymnaea stagnalis* and partially purified α1,3-FucT from human milk.[16] More recently, the cloning of a few helminth-type glycosyltransferases has been reported, including the β1,4GalNAcT from *C. elegans* that we have used here to synthesize the LDN structure [20, 30-32]. In this report we show that the human α1,3-fucosyltransferase FucT-VI is a useful catalyst in the synthesis of the LDNF structure. Our data demonstrate that FucT-VI has a relatively high affinity for LDN as an acceptor. The estimated K_m of 0.27 mM of FucT-VI for LDN-C_8 closely resembles the K_m values of 0.22 mM and 0.32 mM estimated previously for the conventional acceptor LN-C_8 [23, 24], which contains the same C_8 aglycon structure. These data indicate that LDN is as good an acceptor as LN, whereas the K_m value observed for H-type 2 acceptor is slightly lower, as observed previously by De Vries et al.[24, 26] These data support the earlier findings that modification at the C2 of galactose does not hinder, or even may enhance the affinity of FucT-VI for its acceptor substrate. Remarkably, our data show that the nature of the hydrophobic spacer greatly affects the K_m of FucT-VI for its substrate, as demonstrated by the 10-fold increase in K_m value for both the substrates LDN-DAP and LN-pNP. Whereas the latter substrates both contain a hydrophobic aglycon, a long hydrophobic stretch directly connected to the reducing end of GlcNAc is lacking in these acceptors which may explain the reduced affinity for the enzyme FucT-IV.

The observation that human α1,3fucosyltransferases are capable of efficiently synthesizing LDNF, suggests that these epitopes could be present in human cells when FucT-VI is co-expressed with one of the human β1,4GalNAcTs described.[33, 34] There are only a few reports that describe the presence of LDN or LDNF in human glycoproteins, suggesting that their presence is very limited (reviewed in [35]). The most likely explanation of this limited presence seems a limited activity or expression of the human β1,4GalNAcTs responsible for synthesis of the LDN structure, which has to be present before FucT-VI can act.

Multivalency of glycoconjugates is an important property that may greatly influence the biological activity of specific glycan molecules. It has been reported previously,[19] that the linkage of DAP to oligosaccharides allows the subsequent coupling to maleimide-activated proteins. We showed here that linkage of diethyl squarate to DAP-derivatized oligosaccharides prior to linkage to protein offers an attractive alternative coupling strategy. An advantage is that the protein does not need to be activated prior to use so any protein can be used as a carrier. The procedure could be controlled by varying the amount of oligosaccharides

to protein, allowing the coupling of defined amounts of oligosaccharides to the carrier protein.

Summarizing, we described a straightforward method to synthesize the neoglycoconjugate BSA-LDNF, and the methodology described here can be easily applied for the synthesis of other neoglycoconjugates. The availability of neoglycoconjugates with different average number of glycans per carrier allows the evaluation of glycan density and importance of the carrier molecule for biological activity of the neoglycoconjugates.

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CHAPTER 4

Regulation of expression and secretion of galectin-3 in human monocyte-derived dendritic cells

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Abstract

Galectin-3 (Gal-3) is a β-galactoside binding lectin displaying both intracellular and extracellular functions. In *Schistosoma mansoni* infection, Gal-3 has been associated with the induction of a T helper 2 (Th2) response. Whereas dendritic cells (DCs) play a pivotal role in the regulation of T cell differentiation, little is known about the regulation of Gal-3 expression in DCs. In this study we determined Gal-3 mRNA and protein levels during *in vitro* differentiation of human monocytes into immature DCs (iDCs), by culturing monocytes in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Gal-3 mRNA levels show a moderate, transient increase during iDC generation, accompanied by elevated cell-associated Gal-3 protein. Our data showed that culturing monocytes with IL-4 alone strongly increases Gal-3 mRNA levels, whereas GM-CSF induces a low increase in Gal-3 mRNA. The combined data indicate that GM-CSF reduces IL-4 induced Gal-3 mRNA levels during the generation of iDC. Remarkably, stimulation of monocytes with GM-CSF results in secretion of significant amounts of Gal-3 in the medium, whereas iDCs do not release detectable amounts of Gal-3, indicating a suppressive role of IL-4 on GM-CSF induced Gal-3 secretion. Finally, our data demonstrate that all differentiated cell types tested show a significantly lower capacity to bind Gal-3 on the cell surface than monocytes. In conclusion, Gal-3 expression in iDCs is restricted, and Gal-3 protein is localized mainly intracellular, due to the opposite actions of IL-4 and GM-CSF. By these properties, the DCs may be protected against Gal-3 induced phosphatidylserine (PS) exposure and/or apoptosis.

Introduction

The innate immune system is able to detect and eliminate a large array of pathogens. This response is facilitated by the recognition of pathogen-associated molecular patterns (PAMPs), which include a variety of structures selectively expressed by pathogens. Dendritic cells (DC) play a key role in infectious diseases by regulating immune responses upon pathogen recognition. They express a variety of receptors recognizing PAMPs such as Toll like receptors and lectin receptors, which facilitate antigen uptake and presentation, and the induction of pathogen-specific adaptive immune responses [1]. Pathogen-associated glycan structures play an important role in modulation of host immune responses [2-4] by targeting host lectin receptors. Today, many lectin receptors that can recognize pathogen-associated glycans have been identified, including members of the galectin and C-type lectin families[3].

Galectin-3 (Gal-3) is a member of the galectin family of glycan binding proteins sharing the affinity for β-galactosides and significant sequence similarities in their carbohydrate-binding domains [5-7]. Gal-3 is composed of a C-terminal carbohydrate-recognition domain (CRD) and an N-terminal domain containing multiple PGAYPG repeats, allowing multimer formation [8]. Many cells, including myeloid cells, express Gal-3 and the protein has been found intracellular, as well as extracellular, either free or cell bound [9]. The expression and secretion of Gal-3 in macrophages is well documented [9-12].

Gal-3 plays important roles in immune responses to pathogens, for example the induction of leukocyte chemotaxis [13], phagocytosis by macrophages [14], or IgE-mediated cytotoxicity of eosinophils to schistosomes [15]. Gal-3 has been shown to bind to *Leishmania major* surface lipophosphoglycans, leading to the cleavage of Gal-3 into a truncated form that has lost its potential to oligomerize and by this way the parasite inactivates Gal-3 function [16]. We previously demonstrated that during infection with the helminth parasite *Schistosoma mansoni,* high levels of Gal-3 are found in granuloma tissue formed around eggs that are trapped in the liver [17] where it possibly is involved in phagocytosis by macrophages, or in granuloma formation [17-19]. The latter may be hypothesized since LDN (LacdiNAc, GalNAcβ1-4GlcNAc)-coated beads implanted in the liver of healthy mice can induce granuloma formation, in contrast to beads coated with other glycans [20]. In line with this hypothesis is the finding that Gal-3 knock out mice, infected with *S. mansoni*, have reduced granuloma formation and a more pronounced T helper 1 response compared to wild-type mice that show a chronic T helper 2 response after egg-laying [21, 22]. Whereas DCs play a key role in shaping T helper cell responses in infection, the regulation of expression and secretion of Gal-3 in human DCs is not known.

In this study we determined factors that regulate Gal-3 expression and secretion in human dendritic cells *in vitro*, using monocyte-derived DC. The results indicate that expression and secretion of Gal-3 in monocyte-derived immature DCs is tightly regulated, due to the opposite actions of interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

Materials and methods

Antibodies and reagents

The monoclonal antibody (mAb) B2C10 is a Mouse-anti-Human Gal-3 derived from BD biosciences (New Jersey, USA) and polyclonal Goat-anti-Human Gal-3 antibody (sc-192830) and Goat-anti-human β-actin came from Santa Cruz Biotechnology (California, USA). Anti-MGL antibody (mAb 18E4) and anti-DC-SIGN (mAb AZN-D1) were kind gifts from Dr. S. van Vliet and Dr. Y. van Kooyk, respectively [23, 24]. Rabbit anti-Goat IgG (H+L)-alkaline phosphatase and Rabbit-anti-Mouse IgG1-PE were obtained from Zymed laboratories Inc.(California, USA), Rabbit-anti-Mouse (IgG1)- alkaline phosphatase and alkaline phosphatase-anti-streptavidin and streptavidin-peroxidase were from Sigma-Aldrich (Missouri, USA), streptavidin-PE from BD Biosciences and Donkey-ant-Goat IgG(H+L) Alexa fluor 488 from Invitrogen (Oregon, USA). Neoglycoconjugates consisting of Galβ1-4GlcNAc (LN, LacNAc), LDN, β-GalNAc or α-GalNAc multivalently coupled to polyacrylamide (PAA) were purchased from Lectinity (Lappeenranta, Finland). The vector pAN4, and plasmid PET3C-Gal-3, encoding human Gal-3, were kind gifts from Dr. K. Drickamer (London, UK), and Dr. H. Leffler (Lund, Sweden), respectively.

Recombinant Gal-3 production

To produce a full length recombinant human Gal-3 containing a biotin molecule linked to its N-terminus, the cDNA sequence of Gal-3 was amplified by PCR, using plasmid pET3C-Gal-3 as a template, with primer A, containing a BamHI restriction site (5'-GGATCCTTATATCATGGTATATGAAGCACTGG-3') and primer B, containing a SacI restriction site (5'-GAGCTCGATGGCAGACAATTTTTCGCTCCATGATGCG-3'). For amplification of a truncated biotinylated form of Gal-3, lacking the N-terminus, primer A was used in combination with primer C, which contains a SacI restriction site (5'-GAGCTCGCCACTGATTGTGCCTTATAACCTGCC-3'). After digestion of the PCR products with BamHI and SacI, the resulting fractions were ligated into the pAN4 vector, to create pAN4-hGal-3 and pAN4-truncated-hGal-3, and transformed into *Escherichia coli* strain BL21(DE3). After plasmid isolation of several transformants, the desired plasmids were selected by restriction analysis and nucleotide sequencing. For the production of recombinant biotinylated Gal-3, the selected BL21 (DE3) clones were grown in LB medium containing 5 μg/ml ampicilline and 2 μg/ml chloramfenicol, 37°C, until an OD 600 of 0.7 - 0.9. Subsequently, 0.4 mM IPTG and 0.05 mM biotin dissolved in 25% NH_3 was added and the culture was incubated for 3 hours at 37°C. The bacteria were lysed and the recombinant in vivo biotinylated Gal-3 forms were purified by lactosyl-sepharose affinity chromatography.

Monocyte isolation and differentiation

Monocytes were isolated from buffy coats from healthy donors (Sanquin, Amsterdam, the Netherlands) by ficoll gradient centrifugation and CD14 selection with MACS magnetic

microbeads (Miltenybiotec, Bergisch Gladbach, Germany), essentially as described [25]. Isolated monocytes were cultured for 5 days in RPMI 1640 containing 10% fetal calf's serum (FCS), 10,000 U/ml penicillin, 10,000 U/ml streptomycin (BioWhittaker, USA), and 10,000 U/ml glutamine (Invitrogen) in the presence of one or a combination of the following components: IL-4 (500 U/ml, Miltenybiotec, Bergisch Gladbach, Germany), GM-CSF (800U/ml, Miltenybiotec).

Flow cytometry

Differently stimulated monocytes (10^5cells/ staining) were washed with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) supplemented with 1% BSA and incubated for 30 min at 4°C with a monoclonal anti- macrophage galactose-type C-type lectin (MGL) antibody, anti-DC-SIGN mAb AZN-D1 (DC-specific ICAM3-grabbing nonintegrin), polyclonal Goat-anti-Human Gal-3 antibody, biotinylated Gal-3 (10 µg/ml), truncated biotinylated Gal-3 (10 µg/ml), or biotinylated tomato lectin (5 µg/ml) (Sigma-Aldrich Chemie, Munich, Germany). The unbound antibodies were washed away, the cells were incubated with a fluorescent labeled secondary antibody and analyzed by flow cytometry on a BD FACScan (Beckton Dickson San Jose, CA).

Enzyme-linked immunosorbent assay (ELISA)

The neoglycoconjugates LN-PAA, LDN-PAA, β-GalNAc-PAA or α-GalNAc-PAA were coated in 50 mM $NaHCO_3$ (5 µg/ml), for 1h at 37°C. Plates were blocked with 1% ELISA grade BSA (Fraction V, fatty acid free; CalBiochem, California, USA) in TSM (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37°C. Subsequently, biotinylated Gal-3 or biotinylated truncated Gal-3, pre-incubated for 1 h at 25˚C with streptavidin-peroxidase, was added. After incubation of 1 h at 37°C and subsequent washing, binding of Gal-3 was evaluated using TMB substrate. The optical density was measured by a spectrophotometer (BD biosciences) at 450 nm.

To detect Gal-3 in the culture medium of differently stimulated monocytes, a Gal-3 capture ELISA was performed according to the manufacturers guidelines (Calbiochem, Nottingham, United Kingdom). In brief, the coated plate, which was provided by the manufacturers, was washed with wash buffer and the supernatant of the culture (in 1/10 dilution) and the polyclonal biotinylated Gal-3 antibody were added (2 h, on an orbital shaker at 200 rpm, RT). Simultaneously, Gal-3 concentration standards were tested. The plate was washed 4 times with wash buffer and incubated for 1 h with Streptavidin-HRP. The reaction was developed by TMB substrate and optical density measured by a spectrophotometer (450nm).

Western blotting

Proteins were separated by SDS-PAGE on a 12.5% gel and subsequently transferred to a nitrocellulose membrane. The Western blots were incubated with a mixture of mouse-anti-Gal-3 mAb and Goat-anti-human β-actin antibodies, followed by a species specific alkaline phosphatase, or incubation with streptavidin-alkaline phosphatase (for the biotinylated

recombinant Gal-3 proteins). Blots were developed with BCIP (X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate)/NBT (4-Nitro blue tetrazolium chloride) (Promega Corporation, Wisconsin, USA), the intensity of the bands was determined by a gel imager (Epi chemi II Darkroom, UVP Laboratory products) and quantified by the computer program Labworks©. To investigate the presence of a biotin label on the recombinant biotinylated Gal-3 forms, 1µg recombinant protein was analyzed. For analysis of secreted Gal-3 protein, 25 µl of cell culture medium (cells grown at 10^6 cells/ml) was concentrated (Savant SC210A, SpeedVac concentrator, Thermo electron) and dissolved in 15 µl sample buffer. For analysis of cell-associated Gal-3, cells were lysed using lysis buffer (50 mM Tris pH 7.4, 0.1% SDS, 1 µl/100 000 cells) and 2 µl cell lysate was taken up in 13 µl sample buffer.

Quantitative real-time PCR

Total mRNA was isolated with a mRNA capture kit (Roche, Switzerland) and reverse transcribed into cDNA with a reverse transcription system (Promega Corporation) according to the manufacturers guidelines. In brief, cells were washed in ice–cold PBS, harvested by centrifugation and lysed (40 µl/10^5 cells). Lysates were incubated with biotin-labeled oligo (dT) 20 for 5 min at 37°C and then transferred to a streptavidin –coated tube and incubated for 5 min at 37°C. After washing, transcription mix (5mM $MgCl_2$, 1x reverse transcription buffer, 1 mM dNTP, 0.4 U of recombinant Rnase inhibitor Rnasin, 0.4 U of reverse transcriptase, 0.5 µg of random hexamers in nuclease-free water) was added to the mRNA and incubated for 10 min at room temperature and for 75 min at 42°C. To inactivate the transcriptase and separate mRNA from the cDNA, samples were heated for 5 min at 99°C.

Real-time PCR was essentially performed as described [26]. Primers were designed using the Primer Express 2.0 (Applied Biosystems, California, USA) and synthesized by Invitrogen Life technology (Sequence GAPDH: Fwd 5'CCATGTTCGTCATGGGTGTG, Rev 5' GGTGCTAAGCAGTTGGTGGTG; Gal-3 Fwd 5' CAAAGAGGGAATGATGTTGCC, Rev 5' TGTTCTCATTGAAGCGTGGGT). PCR was performed using a SYBR Green based quantitative fluorescence method in an ABI 7900HT sequence detection system (Applied Biosystems). The reaction was carried out in a 96-well optical plate by mixing 4 µl of the two times concentrated SYBR Green Master Mix (Applied Biosystems) with 2 µl of 5 nmol/µl primer solution and 2 µl of the synthesized cDNA product. The profile for the reactions was 2 min at 50°C, followed by 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The Ct value was defined as the number of PCR cycles where the fluorescent signal of SYBR Green exceeded the threshold of 0.2 relative units. The endogenous reference gene was GAPDH.

Results

Characterization of the monocyte-derived cell types

To analyze the regulation of expression of Gal-3 in human monocyte-derived cells, monocytes, freshly derived from healthy individuals, were stimulated with IL-4 and/or GM-CSF to

obtain immature dendritic cells (iDCs), or monocytes stimulated with either GM-CSF or IL-4. To asses whether the expected cell-types were obtained, the cells were analyzed for their expression profile of the C-type lectins DC-SIGN and MGL. DC-SIGN is commonly found on cells of the DC lineage and MGL is expressed on DCs and specific subsets of macrophages. The data show that stimulation of monocytes for 5 days with GM-CSF and IL-4 resulted in the upregulation of the expression of both DC-SIGN and MGL, which is reported as a phenotypical characterization of iDCs [27]. Monocytes stimulated for 5 days with IL-4 alone, have an iDC like phenotype, and show similar expression levels of MGL but a decreased expression of DC-SIGN in comparison to the iDC. By contrast, monocytes stimulated with GM-CSF alone have a macrophage like phenotype, illustrated by upregulated MGL expression, and no expression of DC-SIGN (Fig. 1)[27].

Stimulation with IL-4 enhances Gal-3 mRNA levels in human monocytes

To determine the relative expression levels of Gal-3, mRNA levels of Gal-3 and GAPDH as an endogenous reference, were measured by real-time PCR in monocytes, iDCs and monocytes stimulated with IL-4 or GM-CSF. The data show that Gal-3 mRNA levels measured in monocytes are relatively low (Fig. 2). Monocytes stimulated with IL-4 show significantly higher (p-value = 0.03) Gal-3 mRNA levels than cells stimulated with GM-CSF, or with a combination of GM-

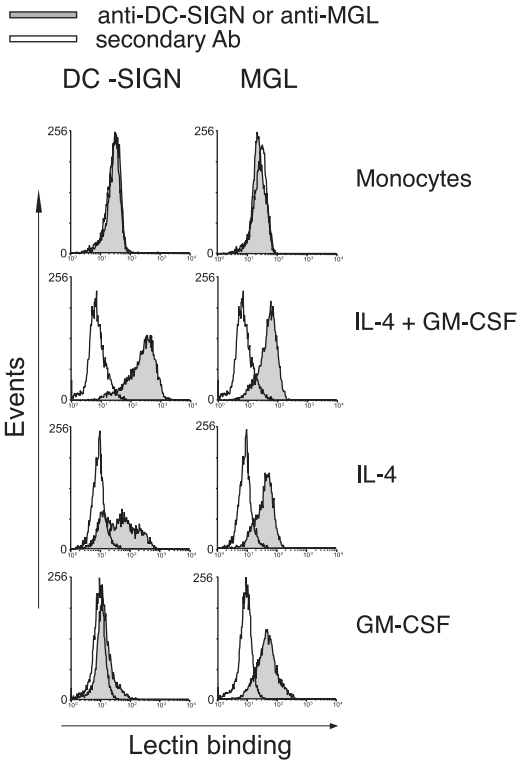

Figure 1. Characterization of differently stimulated monocytes.

Human monocytes were incubated with IL-4 and GM-CSF, as indicated, to create iDCs, or with IL-4 or GM-CSF alone to assess the effect of a single compound on the iDC phenotypes. After 5 days of incubation, the cells were characterized by FACS analysis for the expression of DC-SIGN and MGL, using anti-DC-SIGN and anti-hMGL mAbs. The grey figures show monocytes stimulated with the compound indicated. The open figures represent cells stained with the secondary Ab. The shown results here are representatives of 5 experiments using monocytes isolated from different donors

CSF and IL-4 (Fig. 2). These data suggest that GM-CSF does not significantly affect Gal-3 mRNA expression, but acts as a negative regulator on IL-4-induced Gal-3 mRNA expression.

To determine whether the differences in Gal-3 mRNA levels in the cells are also observed at the protein level, the cells were lysed and total Gal-3 protein levels were estimated by Western blotting, and compared to the levels of actin protein as an intracellular control. The results show that the Gal-3 protein levels in the various differentiated cells are all higher than those in monocytes (Fig. 2). Remarkably, no significant differences were observed in Gal-3 protein levels between the differently stimulated monocytes, despite the observed differences in mRNA levels. These data raised the question whether Gal-3 protein may have been secreted by the IL-4 stimulated monocytes.

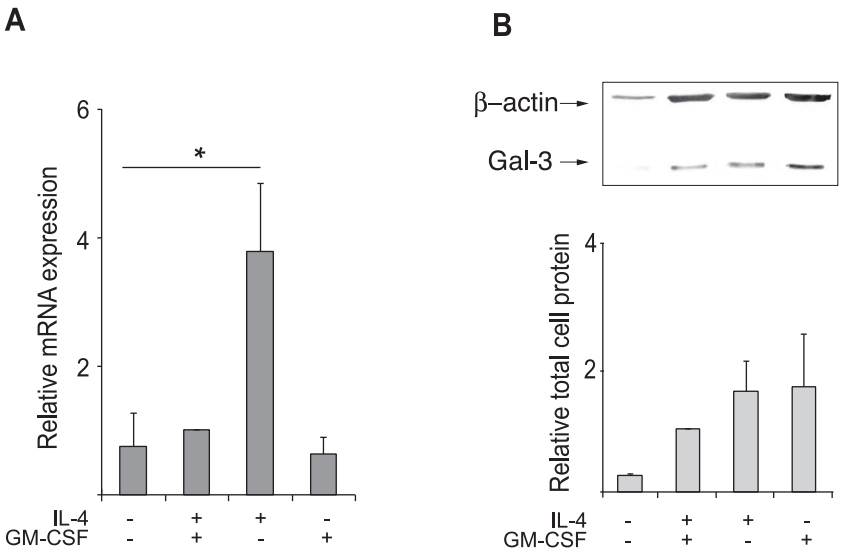

Figure 2. Gal-3 mRNA expression and total Gal-3 protein in cell lysates.
Human monocytes were incubated with IL-4 and GM-CSF to create immature dendritic cells or with IL-4 or GM-CSF alone. A.) Gal-3 mRNA levels in monocyte-derived cells. Gal-3 mRNA levels in the various cell types was measured by quantitative real-time PCR after 5 days of culturing the monocytes with the stimuli indicated. The mRNA levels are expressed relative to GAPDH mRNA, and the values of the iDC (GM-CSF+IL-4) were set at 1. The results shown here are average values of cells derived from 4-6 different donors. (* p-value< 0.05 compared to monocytes). B.) Gal-3 protein levels in monocyte-derived cells. The total protein content of Gal-3 was analysed in the different cell types, after 5 days of culturing the monocytes with the stimuli indicated. The cells were lysed and the Gal-3 content was determined by Western blotting and quantified by Labworks©. The amount of Gal-3 was corrected for the β-actin content in the cells. The upper part shows a Western blot derived from one representative donor. The lower part shows the average amount of Gal-3 in cell lysates derived from 5 experiments using monocytes from different donors. The average amount of Gal-3 in iDCs was set at 1.

Monocytes stimulated by GM-CSF secrete Gal-3

To define factors regulating secretion of Gal-3 protein from human monocyte-derived cells, the amount of Gal-3 in the medium was analyzed by Western blotting, after culturing the monocytes for 5 days with IL-4, GM-CSF or a combination of these stimuli. To enable correction for Gal-3 in the medium that may be derived from cell lysis, the amount of β-actin as an intracellular control was measured simultaneously. Unexpectedly, only the growth medium of monocytes stimulated with GM-CSF alone contained significant levels of Gal-3 (p-value= 0.02, compared to iDCs) (Fig.3). In the growth media of IL-4 stimulated monocytes, or iDCs that were obtained by culturing monocytes with IL-4 and GM-CSF, no Gal-3 has been detected in any of the donors tested. These data indicate that GM-CSF allows release of Gal-3 into the medium.

Since no increase in Gal-3 mRNA levels was detected after 5 days of culturing monocytes with GM-CSF, compared to the levels in untreated monocytes, the possibility was explored that GM-CSF induces a transient upregulation of mRNA expression, followed by secretion of the protein. Therefore, the mRNA levels of Gal-3 in monocytes stimulated with GM-CSF, and the amount of Gal-3 protein secreted by these cells, were measured. The data were compared with those for cells stimulated with IL-4, and for iDC, with a one-day interval over the total time course of differentiation (Fig. 4). After 24 hours of culture, a major increase of Gal-3 mRNA levels in the IL-4 stimulated monocytes and, to a lower degree, in the cells stimulated with both GM-CSF and IL-4 was observed (Fig. 4A). The level of Gal-3 mRNA in GM-CSF-stimulated monocytes was lower at all time points than those of the other cell types. However, a transient increase in mRNA level was observed during the differentiation period with GM-CSF, which is the highest on day 2. Again, only in the medium of GM-CSF-stimulated monocytes Gal-3 protein is detected starting at day 3, and an increased amount is detected at days 4 and 5 (Fig. 4B). In conclusion, the data indicate that IL-4 induces the expression of high levels of

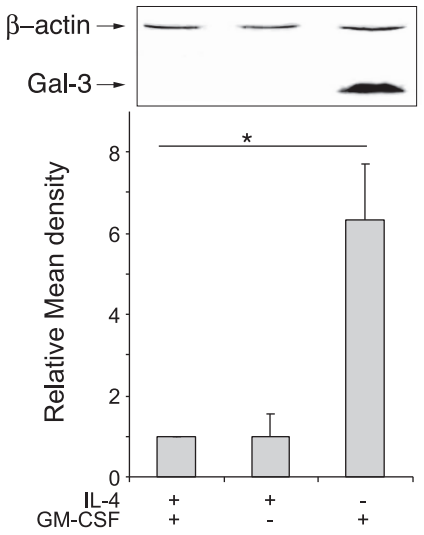

Figure 3. Gal-3 protein in the culture medium of different cell types.

Human monocytes were cultured with IL-4 and GM-CSF or with IL-4 or GM-CSF alone, as indicated. After 5 days, the medium of the differently stimulated monocytes was tested for their Gal-3 content via Western blot and Gal-3 amounts were quantified via Labworks©. The amount of β-actin was measured to correct for Gal-3 derived from cell lysis. The upper part shows a Western blot with the results of different cell types derived from one representative donor. The lower part shows the average values derived from 5 donors and the total amount of Gal-3 estimated in iDC was set at 1 (* p value < 0.05 compared to iDC).

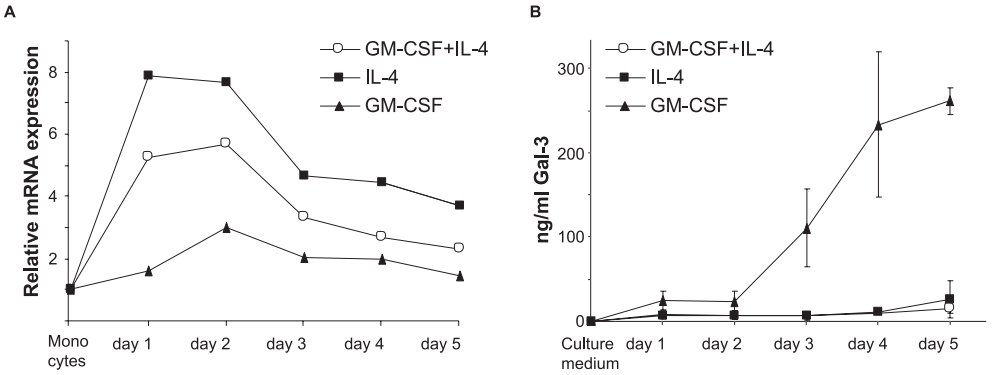

Figure 4. GM-CSF induces low Gal-3 mRNA expression in monocyte-derived cells and triggers secretion of Gal-3 protein.

A.) Timecourse of Gal-3 mRNA levels of monocytes cultured with IL-4 and GM-CSF to create iDCs, or with IL-4 or GM-CSF alone. mRNA levels were measured by Real-time PCR daily, starting at the day of isolation of the monocytes until day 5 of incubation. Gal-3 mRNA values are expressed relative to GAPDH and the values of the monocytes were set at 1. The data shown is a representative experiment out of 3 (different donors), performed in duplicate. B.) Time course of secretion of Gal-3 protein in the culture medium of monocytes cultured with IL-4 and GM-CSF to create iDCs, or with IL-4 or GM-CSF alone. The amount of Gal-3 protein was estimated in the culture medium of the different cell types used in A, every 24 hours after start of the incubation until day 5, by a Gal-3 capture ELISA. The experiment shows the average values derived from 3 different donors.

Gal-3 mRNA. By contrast, GM-CSF induces a much lower transient Gal-3 mRNA expression and triggers secretion of Gal-3 protein into the medium. In iDCs, which have been stimulated by both IL-4 and GM-CSF, intermediate Gal-3 mRNA levels are detected and no or very l ow Gal-3 levels are detected in the medium suggesting that either the translation of mRNA to protein, and/or the secretion of Gal-3 into the medium is hampered by IL-4 signaling.

Localization of Gal-3 at the cell surface

The results in Fig. 2 show no significant differences in the estimated Gal-3 protein levels in monocytes stimulated with either IL-4, GM-CSF or both IL-4 and GM-CSF. However, all differentiated cell types showed enhanced Gal-3 levels compared to monocytes, suggesting that additional Gal-3 had been synthesized, which can be present either intracellular or bound at the cell-surface. To explore the possibility that Gal-3 is localized at the cell surface, the surface bound Gal-3 was measured by flow cytometry. The data showed that monocytes have a significant higher amount of surface-bound Gal-3 than iDCs (p-value= 0.002, compared to iDC). In the other cell-types, a low amount of Gal-3 was detected at the cell-surface in some donors (Fig. 5A). This indicates that Gal-3 disappears from the cell surface after differentiation of the monocytes. The data showed that a strong decrease of surface bound Gal-3 is already

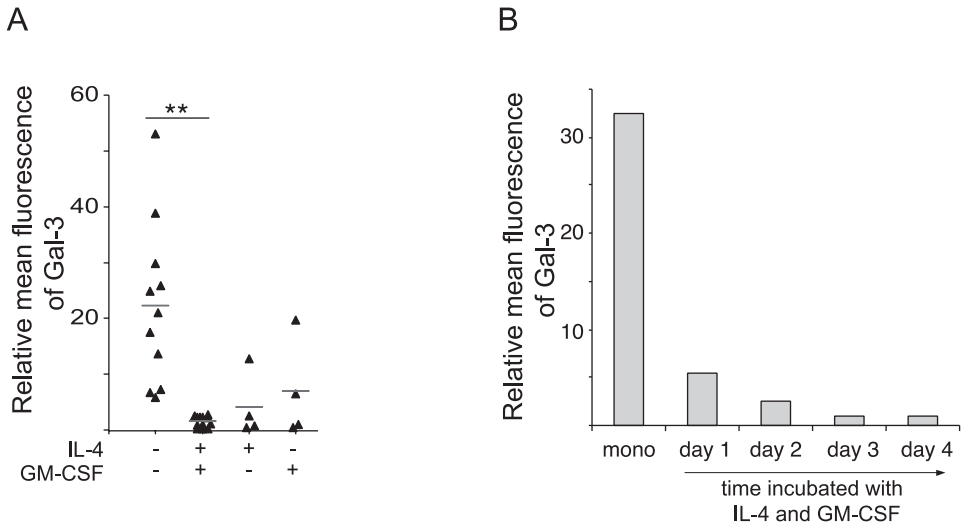

Figure 5. Monocytes have high levels of surface bound endogenous Gal-3, which strongly decreases upon differentiation to iDCs.

Monocytes, and monocytes cultured for 5 days with IL-4 and/or GM-CSF as indicated, were analyzed for surface expression of Gal-3 by an anti-Gal-3 antibody via FACS analysis. The relative mean fluorescence is obtained by dividing the mean fluorescence of the anti-Gal-3 binding by the mean fluorescence of the unstained cells. A.) Monocytes have high levels of surface bound endogenous Gal-3. Surface bound endogenous Gal-3 was measured on monocytes directly upon isolation. For the other cell-types, surface Gal-3 was measured after 5 days culturing in the presence of IL-4 and/or GM-CSF, as indicated. Data are shown from duplicate measurements from cells derived from al least 3 different donors. (** p-value< 0.01 compared to iDC). B.) Surface bound endogenous Gal-3 decreases upon differentiation of monocytes to iDCs. Surface bound endogenous Gal-3 was measured on monocytes directly upon isolation, and daily upon incubation with IL-4 and GM-CSF during the culture period. The Figure shows a representative experiment out of 3 experiments, performed in duplicate, with monocytes derived from different donors.

observed after 24 hours of stimulation with GM-CSF and IL-4, which may indicate that the monocyte cell surface ligands for Gal-3 are lost within one day (Fig. 5B).

Production of recombinant biotinylated Gal-3

The differences between the amount of surface-bound Gal-3 between monocytes and the differentiated cell-types could reflect differences in the presence of surface receptors (ligands) for Gal-3, or the external presence of Gal-3. To discriminate between the amount of surface-bound Gal-3, and the ability of the cells to bind Gal-3, we examined whether the different cell types have the potential to bind externally added Gal-3. Therefore, a recombinant full-length form of Gal-3, and a truncated form of human Gal-3 containing a CRD but no N-terminal domain, were produced. Both Gal-3 forms were biotinylated *in vivo* at their N-terminus. The

integrity and purity of the produced proteins was established by SDS-PAGE analysis, Western blotting and ELISA (Fig. 6). SDS-PAGE analysis showed that highly pure proteins with apparent MWs of 30 kDa for the full length and 17 kDa for the truncated form of Gal-3 were produced (data not shown). In Western blots these forms were recognized by anti-Gal-3 mAbs and streptavidin (Fig. 6A). To examine the carbohydrate binding capacity of the recombinant Gal-3 proteins, they were tested for binding to known ligands by ELISA. Both Gal-3 forms bound to glycoconjugates containing LN and LDN structures, respectively (Fig. 6B), whereas no binding to control glycoconjugates was observed. These data collectively indicate that active biotinylated recombinant forms of Gal-3 have been produced.

Binding of externally added Gal-3 to the cell surface of monocyte-derived cells

To assess the presence of free receptors for Gal-3 on the cell surface, the cells were incubated with biotinylated Gal-3 and analyzed by flow cytometry. The data (Fig. 7) show that monocytes strongly bind externally added Gal-3 (p-value= 0.03, compared to iDC), whereas all other cell types showed no significant binding of Gal-3 with the exception of two donors (Fig. 7). This suggests that monocytes have a high amount of Gal-3 receptors on their cell surface, in contrast to all other cell-types tested. The binding of externally added Gal-3 to monocytes is carbohydrate-dependent since they could bind both the truncated and the full length Gal-3, and addition of 150 mM lactose inhibited binding of both Gal-3 forms (data not shown).

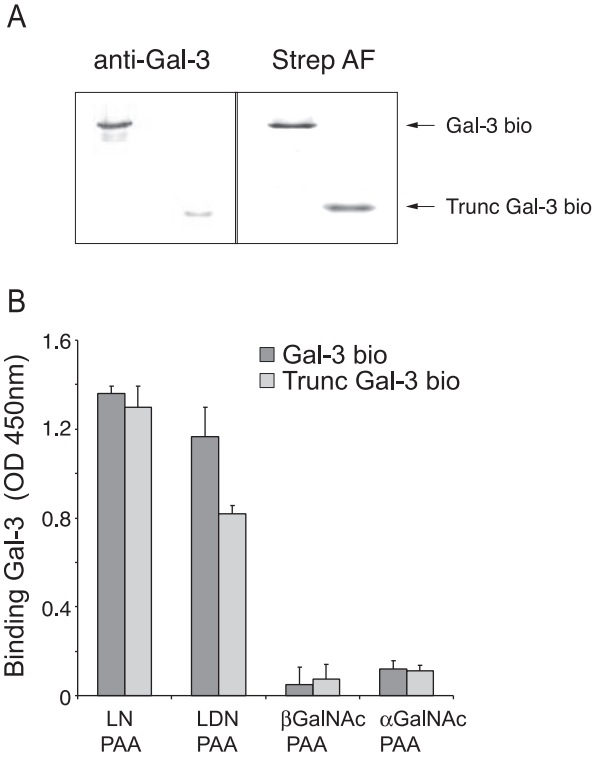

Figure 6. Characterization of biotinylated recombinant Gal-3 and truncated Gal-3.

A.) Produced recombinant forms of Gal-3 and truncated Gal-3 were characterized by Western blotting, using anti-Gal-3 Abs and streptavidin-AF, respectively. B.) Both recombinant biotinylated Gal-3 forms bind to neoglycoconjugates containing the ligands for Gal-3 (LN-PAA and LDN-PAA, coated at 5 µg/ml), but not to control glycoconjugates (α/βGalNAc-PAA, coated at 5 µg/ml), as determined by ELISA.

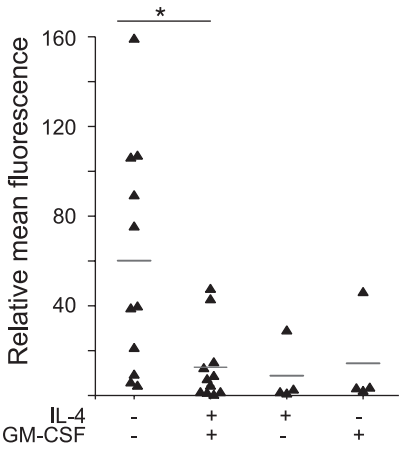

Figure 7. Monocytes bind significant more externally added biotinylated Gal-3 than monocyte-derived cell-types obtained by culturing with IL-4 and/or GM-CSF. Monocytes bind on average more externally added biotinylated Gal-3 (full-length form) than monocyte-derived cells that were obtained by culturing for 5 days with IL-4 and GM-CSF or with IL-4 or GM-CSF alone, as determined by FACS analysis. Similar results were obtained using the truncated Gal-3 form (not shown). The mean fluorescence obtained with biotinylated Gal-3 was divided by the mean fluorescence of unstained cells for each measurement (* p-value< 0.05 compared to iDC).

Remarkably, a large individual variability was observed between monocytes derived from different donors, both in the presence of endogenous Gal-3 on the surface (Fig. 5), as well as in the capacity of the monocytes to bind additional externally added Gal-3 (Fig. 7). Comparison of the two properties per individual donor revealed that monocyte preparations that showed high levels of bound endogenous Gal-3, could not bind much additional Gal-3-bio when added, and vice versa (not shown). These data indicate that all monocytes can bind significant amounts of Gal-3.

Discussion

Gal-3 has been implicated as a key regulator in innate and adaptive immune responses. Gal-3 and other galectins are soluble proteins that often act as extra-cellular immune modulators. The cells synthesizing and secreting these proteins may differ from the cells targeted by galectins. Myeloid cells have been shown previously to synthesize and secrete Gal-3 [9]. Monocytes have been reported to express low levels of Gal-3, and differentiation of human monocytes progenitors, HL-60 cells, into macrophages resulted in significantly increased levels of Gal-3 mRNA and protein [28, 29]. To increase the understanding of the regulatory roles of Gal-3 in immune responses, we here report how the synthesis and secretion of Gal-3 in human monocyte-derived DCs is regulated.

To date, the factors regulating Gal-3 mRNA expression in human monocyte-derived cells are incompletely understood. It has been reported that Gal-3 expression in the monocyte/macrophage pathway is partly regulated at the posttranscriptional level, by stabilization of its mRNA [29]. Furthermore, by gene array studies it has been shown that Gal-3 is expressed by iDCs, and decreases upon maturation of the DCs [30]. Using real-time PCR, and monocyte-derived cells cultured under defined conditions differing only in the cytokines added to induce their differentiation, we confirm earlier data that monocytes contain relatively low levels of Gal-3 mRNA. Differentiation of monocytes to iDCs by culturing for 5 days in the presence

of IL-4 and GM-CSF induces a moderate increase in Gal-3 mRNA levels. However, culturing the monocytes with IL-4 alone, inducing a DC-like phenotype [27], results in strongly increased mRNA levels, in contrast to GM-CSF that only induces a low transient increase in Gal-3 mRNA levels. The fact that the combination of IL-4 and GM-CSF results in reduced Gal-3 mRNA levels suggests that during culture to iDCs, GM-CSF suppresses IL-4-induced Gal-3 mRNA expression. Thus, the moderate levels of Gal-3 mRNA in iDCs appear to be the result of the opposite effects of IL-4 and GM-CSF.

The striking differences in Gal-3 mRNA expression observed for the IL-4-treated monocytes in comparison to the other cell types, were not as pronounced at the protein level. The moderate increase that we observed in total cellular Gal-3 protein of IL-4 treated monocytes is in agreement with a recent report [10]. In the latter report the authors demonstrated also an IL-4 induced secretion of Gal-3, which was not found in this study and may be explained by differences in the culture media, which may contain factors that affect release of Gal-3. Unexpectedly, considerable amounts of Gal-3 were found in the medium of GM-CSF treated monocytes. These cells showed only a low, transient upregulation of Gal-3 mRNA levels after 24-48 hours of culture, directly followed by secretion of Gal-3 protein in the medium. This indicates that GM-CSF can induce release of Gal-3 into the medium. Interestingly, iDCs, which have been cultured with a combination of GM-CSF and IL-4, hardly showed Gal-3 release into the medium. Apparently, IL-4 inhibits GM-CSF-induced release of Gal-3. In summary, both Gal-3 expression and secretion appears to be restricted in iDCs, by the concerted, opposite actions of GM-CSF and IL-4.

Our data and data from other groups suggest that the secretion of Gal-3 is highly dependent on the system used, indicating the presence of unknown factors needed to stimulate Gal-3 release. For instance some mouse macrophages (J774.2 cells) have been reported to secrete 30-45 % of total lectin content, whereas WEHI-3 cells have been reported to secrete little if any Gal-3 [31] . A role for GM-CSF in increasing extracellular Gal-3 levels has been suggested previously [32]. The authors showed that nerve lesions cause fibroblasts to produce GM-CSF, which in turn induces Schwann cells and macrophages to up-regulate surface expression of Gal-3. Using defined conditions, we now have demonstrated that the presence of GM-CSF can induce the release of Gal-3, rather than an increase in expression. GM-CSF is an important haematopoietic growth factor with immune modulatory properties, which is secreted by a variety of cells including T-cells and macrophages, and has the capacity to recruit circulating monocytes, neutrophils and lymphocytes [33]. It may be possible that in environments where GM-CSF recruits monocytes, these monocytes are induced to produce and secrete Gal-3 which on its turn is also a chemoattractant for monocytes and macrophages [13] facilitating a broad influx of inflammatory cells. Such a role for Gal-3 may also be hypothesized in schistosoma-induced liver granuloma, where large amounts of Gal-3 have been demonstrated [17].

Whereas all monocyte-derived differentiated cells tested contained cell-associated Gal-3 protein, clear differences were found in the amount of surface-bound Gal-3, and the potential of these cells to bind externally added Gal-3. Our data suggest that monocytes have a high

capacity to bind Gal-3, in contrast to the differentiated cell types tested here. Remarkably, a large variation was observed in the amount of cell-surface bound Gal-3 upon isolation of the monocytes. Monocytes that showed low Gal-3 levels at their surface, could bind more externally added Gal-3 than monocytes that already showed high surface Gal-3 levels at the time of isolation, and vice-versa. These data suggest that all monocytes have the capacity to bind Gal-3 and that monocytes from some individuals already carry surface Gal-3 at the time of isolation, which may be derived from the serum of these individuals. On monocytes, Gal-3 may play a role in mediating homotypic aggregation via CD13, which could lead to multinucleated giant cell formation, a phenotypical characterization associated with alternatative macrophage activation and chronic inflammation [34]. Here we show that upon differentiation to iDCs, Gal-3 disappears for more than 90% from the cell-surface within 24-48 hours of culture. Compared to monocytes, iDCs also show a greatly reduced capacity to bind externally added Gal-3, or tomato lectin (not shown), indicating that Gal-3 receptors on the cells are lost during IL-4/GM-CSF induced differentiation. It has been shown that Gal-3 primarily recognizes poly-N-acetyllactosamine [poly(LN)]-containing glycans, which can be detected by tomato lectin, and can bind to both internal and terminal LN within poly (LN) [35, 36]. In monocytes, Lysosome associated membrane proteins 1/2 (LAMP1/2) are reported to contain poly (LN) that can function as ligands for Gal-3 [37]. The lysosomal membrane proteins LAMP1/2 are partly surface localized, and may be involved in a fast shuttling of Gal-3 toward the endosomal/lysosomal compartment upon differentiation. In addition, the glycosylation potential of the cells may change during differentiation of monocytes to iDCs.

Summarizing, we demonstrate that Gal-3 expression in monocyte–derived iDC is tightly regulated by the opposite actions of GM-CSF and IL-4. Several galectins, including Gal-3 have been shown to induce phosphatidylserine (PS) exposure in activated leukocytes, which targets the cells for phagocytosis by macrophages [38-40], an important mechanism to restore homeostasis after an inflammatory period. In addition, Gal-3 has been shown to induce apoptosis in T cells [40]. The low capacity of iDC to bind Gal-3 may prevent a Gal-3-induced PS exposure and apoptosis of these cells, which would be in line with their function as surveyors of the immune system. In this study however, only the binding of Gal-3 to iDCs has been explored and it cannot be excluded that other galectins do bind and functionally affect these cells, which should be examined in future studies.

Acknowledgements

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CHAPTER 5

Schistosoma mansoni worm glycolipids induce an inflammatory phenotype in human dendritic cells by simultaneous engagement of TLR4 and the C-type lectin DC-SIGN

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Abstract

The human helminth parasite *Schistosoma mansoni* induces a Th1 immune response early in infection, which shifts to a dominant Th2 response after egg-laying. To increase our understanding of the mechanisms that play a role in shaping the host immune responses, we have investigated the effects of schistosome glycoconjugates on the phenotype of dendritic cells (DC). DCs play a crucial role in the regulation of the immune response and the linking of innate and adaptive immunity. DCs detect invading pathogens using pathogen recognition receptors such as Toll-like receptors and C-type lectins. We show here that *S. mansoni* worm glycolipids induce activation of DCs as deduced from the upregulation of maturation markers and the production of pro-inflammatory cytokines. Co-culture of glycolipid-primed DCs with naïve T cells results in skewing of the T cell response towards a T helper cell type 1 profile. Glycolipid-induced DC maturation is dependent on TLR4 signalling and requires intact glycan-moieties, including Galβ1-4(Fucα1-3)GlcNAc- (Le^X) and GalNAcβ1-4(Fucα1-3)GlcNAc- (LDNF), on the glycolipid species. Although, the maturation process is TLR4 dependent, the worm glycolipids cannot trigger activation of TLR4 when expressed in HEK293 cells. Remarkably, co-transfection of the C-type lectin DC-SIGN and TLR4 in the HEK293 cells facilitated TLR4 signaling by worm glycolipids. Our data demonstrate that worm glycolipids induce activation of DCs via a novel mechanism, requiring collaboration between the DC receptors DC-SIGN and TLR4.

Introduction

The helminth trematode *Schistosoma mansoni* is one of the causative agents of *schistosomiasis,* which is the second most prevalent human parasitic disease worldwide after malaria [1,2]. Infection with *S. mansoni* is initiated when cercariae released from their intermediate host, a snail of the genus *Biomphalaria*, penetrate the skin of the mammalian host. In the host, the cercariae transform into schistosomula, which enter the vasculature and mature to adult worms that produce hundreds of eggs daily over a live span of 5-30 years. Many eggs are secreted by the host into the environment, but part of the eggs end up in host tissue such as the liver thereby causing granuloma formation and pathology [3].

During infection, the immune system is continuously triggered by an array of molecules associated with the different stages of *S. mansoni.* Schistosome glycoconjungates (glycoproteins and glycolipids) play an important role in host parasite interplay and induce both humoral and cellular immune responses in the host [4-6]. *S. mansoni* synthesizes a tremendous amount of different complex glycan structures. Some of them are shared by the host like Lewis X (Le^x, Galβ1-4(Fucα1-3)GlcNAc)) antigens, which are expressed on all stages of *S. mansoni* [7]. In addition to Le^x, *S. mansoni* expresses high amounts of other fucose-containing glycan epitopes such as Fucα1-3GalNAcβ1-4GlcNAc (FLDN), GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF), and glycans carrying multiple fucose residues including GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) [5,8].

The ability of helminths to survive in their hosts is due to their potential to counteract inflammatory responses induced by the host and thus maintaining a balance between host immune activation and suppression. In the host, schistosome infection starts with an inflammatory T helper 1 (Th1) response, which shifts towards a strong anti-inflammatory Th2 response at the time of egg-laying [9]. Next to Th2 cells also regulatory T cells regulate Th1 development and immunopathology in *S. mansoni* infections [10,11].

The molecular events leading to glycan-induced innate and adaptive host immune responses are far from understood, but should critically involve the innate immune system that recognizes the pathogen via antigen-presenting cells. This recognition is mediated by signaling via pathogen recognition receptors (PRRs), which include large families of Toll like receptors (TLRs) and C-type lectin receptors (CLRs) [12]. The activation of TLRs is induced by a group of pathogen-associated recognition patterns including proteins, carbohydrates, lipids and nucleic acids [13]. Several helminth-derived structures are reported to trigger activation of TLRs. Glycoconjugates containing Lacto-N-fucopentaose III (LNFPIII, a milk sugar carrying a Le^x moiety) induce signalling in murine dendritic cells (DCs) via TLR4 [14]. In addition, TLR2 and TLR3 on DCs are stimulated by respectively lysophosphatidylserine (Lyso-PS) [15] and ds RNA from *S. mansoni* egg [16].

CLRs play an important role in regulating DC functions, by the recognition of pathogen associated glycans. CLRs bind glycan antigens via one or more carbohydrate recognition domains (CRDs) in a Ca^{2+} dependent manner. CLRs generally act as endocytic receptor, but

a number of CLRs also show signaling capacity, which could lead to modulation of TLR-mediated signaling [17-19]. We demonstrated recently that the CLR dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) recognizes Le^X and LDNF glycans on soluble egg antigens (SEA), and Le^X and pseudo-Le^Y glycans on cercarial glycolipids of *S. mansoni* [20-22]. In addition, the CLRs macrophage galactose-type lectin (MGL) and Mannose receptor (MR) recognize glycan antigens of *S. mansoni* [23,24]. Human DCs can bind and internalize *S. mansoni* SEA antigens via the CLRs DC-SIGN, MR and MGL, thereby inducing polarization of the DCs towards a Th2 phenotype [23]. It is supposed that the interactions of dendritic cell C-type lectins with schistosome glycans play a role in modulation of dendritic cell function, however the molecular mechanisms are virtually unknown. In addition, schistosomes have been shown to express a large variety of (stage-specific) glycans linked to proteins or lipids, and they may differentially modulate DC function.

Here we report that DCs, primed with *S. mansoni* adult worm glycolipids, skew T cell responses towards a T helper cell type 1 profile. The worm glycolipids induced a strong maturation of the DC as indicated by upregulation of the maturation markers CD80, CD86 and MHC-II, as well as the production of the cytokines Interleukin-12 p40 (IL-12 p40), IL-10, IL-1β, IL-6, IL-8 and tumor necrosis factor-α (TNF-α). Moreover our data strongly suggest that the glycolipid induced DC activation is dependent on collaborative interaction of DC-SIGN and TLR4.

Materials and Methods

Cells and antibodies

Human monocytes were isolated from buffy coats from healthy donors (Sanquin, Amsterdam, the Netherlands) by ficoll gradient centrifugation and CD14 selection with MACS magnetic microbeads (Miltenybiotec, Bergisch Gladbach, Germany), essentially as described [22]. Dendritic cells were prepared by culturing the isolated monocytes for 5 days in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (respectively 500U/ml and 800 U/ml, Miltenybiotec, Bergisch Gladbach, Germany) in RPMI 1640 containing 10 % fetal calf's serum (FCS), 10,000 U/ml penicillin, 10,000 U/ml streptomycin (BioWhittaker, USA), and 10,000 U/ml glutamine (Invitrogen, Carlsbad, CA, USA). LPS (from *E.coli* 0111:B4 Sigma-Aldrich, St. Louis, USA), 1-10 ng/ml of glycosphingolipids of adult worm *S. mansoni* (worm GL, used in concentrations and treated as indicated) in the presence or absence of DC-SIGN (20 μg/ml) or TLR4 (10 μg/ml) blocking antibodies were added to the DCs at day 5 and incubation was continued for 24 h. DC maturation was assessed by flow cytometric determination of the maturation markers MHC class II, CD80 and/or CD86 (Beckton Dickson biosciences, San Jose, CA). Human embryonic kidney cells 293 (HEK 293 cells) stably expressing TLR4 or TLR2 and HEK 293 cells secreting MD2, a kind gift from D.T. Golenbock (Massachusetts, USA), were maintained in DMEM containing 10% FCS, 10,000 U/ml streptomycin, and 10,000 U/ml glutamine and 0,5 mg/ml Geneticin (G418, Invitrogen). HEK 293-TLR2 and HEK 293-TLR4 were transduced with DC-SIGN as described [25,26] and

sorted on the MoFlo (Beckman Coulter, California, USA) on their expression of DC-SIGN. The DC-SIGN-viral construct [25,26] was kindly provided by M. de Jong and T. Geijtenbeek, VU Medical Center, Amsterdam). Activation of TLR2 and/or TLR4 was assessed by determining production of IL-8 after 24 h of stimulation of the HEK transfectants by Pam3CysK4 (TLR2) or LPS (TLR4 + TLR2), or the glycosphingolipids of adult worm *S. mansoni*. Medium containing MD2 was added to the HEK-TLR4 and the HEK-TLR4-DC-SIGN cells in all experiments. The following antibodies were used: AZN-D1 (anti DC-SIGN) [27], anti-Lewis X (G8G12) [28], anti-LDN (mAb SMLDN1.1) [29], anti-LDNF (Sm LDNF) [30], anti-LDNDF (mAb 114-5B1-A) [31], goat-anti-mouse-IgM-peroxidase (Jackson, West Grove, PA, USA), anti-TLR4 blocking antibody (GenWay Biotech, Inc., San Diego, USA). Neoglycoconjugates containing Le^X or LDN multivalently coupled to polyacrylamide (PAA) were purchased from Lectinity (Lappeenranta, Finland).

Preparation of glycosphingolipid fractions

Glycosphingolipids were obtained from lyophilized *S. mansoni* worms by organic solvent extraction, saponification, desalting and anion-exchange chromatography as described [32]. GL were further fractionated chromatographically. The sample was dissolved in 1 or 5 ml of chloroform, dependent on the amount of GL, and applied on a silica cartridge (Waters, Eschborn, Germany), which was calibrated with chloroform. The different fractions were eluted from the cartridge by chloroform: methanol (C:M) and chloroform: methanol: water (C:M:W). Eluents used were C:M 90:10 (v/v); C:M 80:20 (v/v); C:M 70:30(v/v); C:M 60:40 (v/v); C:M 40:60 (v/v); C:M:W 60:35:8 (v:v:v); C:M:W 30:60:8 (v/v/v); C:M:W 10:70:20 (v/v/v). Each fraction was dried under nitrogen stream and taken up in the according solvent

Defucosylation of schistosome worm glycolipids

Fucose moieties were removed from the worm GL by treatment with 48 % (v/v) hydrogen fluoride (HF) (Merck, Darmstadt, Germany) at 4 °C for 48 h. The HF was completely removed by nitrogen stream and extensive washing with methanol [33]. The degree of defucosylation and the integrity of the remaining glycan antigens was assessed by ELISA using monoclonal antibodies recognizing specific glycan epitopes, and MALDI-TOF-MS analysis to assess the integrity of the ceramides.

The carbohydrate structures were uncoupled from their ceramide part by treatment with endoglycoceramidase II (from *Rhodococcus spp.*,Takara Shuzu Co., Otsu, Shiga, Japan). Released ceramides were collected by reverse-phase chromatography as described by Wuhrer et al. [32] and the absence of glycans was established by MALDI-TOF-MS analysis.

Enzyme-linked immunosorbent assay (ELISA) with anti-glycan monoclonal antibodies

Worm GL (10 ng/well), HF treated worm GL (10 ng/well) and the glycolipid fractions, P1 and P2 (10 ng/well) were diluted in ethanol, sonicated and coated on a NUNC maxisorb

plate (Roskilde, Denmark) for 1 h at 37 °C. SEA, Le^X-PAA and LDN-PAA were coated at a concentration of 5 µg/ml (in 50 mM $NaHCO_3$) for 1 h at 37 °C. Plates were blocked with 1 % ELISA grade BSA (Fraction V, fatty acid free; CalBiochem, San Diego, CA) in TSM (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37 °C. Subsequently, antigens were incubated for 1 h at 37 °C with anti-glycan monoclonal antibodies (mAbs). Binding of the mAbs was detected by goat-anti-mouse-peroxidase. The reaction was developed by TMB substrate and optical density measured by a spectrophotometer at 450 nm.

Cell adhesion assay

NUNC maxisorb 96 well plate was coated with worm GL (10 ng/well), HF treated worm GL (10 ng/well), or the different glycolipid fractions (10 ng/well) in ethanol and dried at 37 °C. Le^X-PAA (5 µg/ml) was coated in 50 mM $NaHCO_3$ for 1 h at 37 °C, and after washing incubated for 30 min at 37 °C with 1 % BSA in TSM (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) as blocking step. Dendritic cells labeled with Calceine AM (25 µl/7 × 10^6 cells; Molecular Probes, Eugene, OR) were added in the presence or absence of 20 µg/ml AZN-D1 (anti- DC-SIGN mAb) for 1.5 h at 37 °C. The non-adherent cells were gently washed away and the adherent cells were lysed (50 mM Tris–HCl, pH 7.4, 0.1 % SDS). The released fluorescence was quantified on a Fluostar spectrofluorimeter (BMG Labtech, Offenburg, Germany) at 485/520 nm. Results are expressed as the mean percentage of adhesion of triplicate wells.

IL-8 cytokine ELISA

To detect IL-8 production by the HEK 293 cells transfected with TLR2 or TLR4 and/or transduced with DC-SIGN, a cytokine ELISA was performed according to the manufacturer guidelines (Biosource international, Inc. CA, USA). Shortly, a NUNC maxisorb 96 well plate was coated with 1 µg/ml IL-8 coating Ab overnight at 4 °C. After blocking for 1 h, the supernatant of the culture (in 1/10 dilution) and the detection Ab (0.04 µg/ml) was added (2 h, on an orbital shaker at 500 rpm, RT). Simultaneously, IL-8 concentration standards were tested. The plate was washed 4 times with PBS and incubated for 30 min with Streptavidin-HRP. The reaction was developed by TMB substrate and optical density measured by a spectrophotometer (450nm).

Multiplex bead immunoassay

Cytokine production was measured by multiplex bead immunoassay, following the manufacturer's manual (Invitrogen). Shortly, antibody conjugated beads specific for IL-12 p40, IL-10, IL-1β, IL-6, IL-8 and TNF-α were inserted in a washed 96 well filter plate and washed. Medium derived after stimulation of DCs with worm GL or controls, or the standards (Human Twenty-Five-Plex) were added to the antibody conjugated beads and incubated for 3 h at room temperature (on an orbital shaker at 500-600 rpm in the dark) in the presence of the specific biotinylated detection antibodies. Medium components and antibodies that were not bound to the beads were washed away and the beads were subsequently incubated with

R-Phycoerythrin conjugate 30 min room temperature (on an orbital shaker at 500-600 rpm in the dark). The amount of cytokine present in the medium was measured by luminex[100] (Bio-Rad live sciences, Hercules, CA) and compared with the standards.

Flow cytometry

DC treated as indicated (10^5 cells) were washed with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) supplemented with 1 % BSA and incubated for 30 min at 4 °C with anti-CD80-PE, anti-CD86-PE or anti-HLA-DR-PE. The unbound antibodies were washed away and the cells were analyzed by flow cytometry on a BD FACSScan (Beckton Dickson San Jose,CA). In all experiments, the relative difference in mean fluorescence intensity was determined in relation to the fluorescence observed by 10 ng/ml LPS.

Dendritic cell driven Th1/Th2 differentiation

Immature DCs were incubated with adult GL (0.75 µg/ml). The following positive controls were included in the assay: (i) for a mixed Th1/Th2 response, 100 ng/ml *E. coli* LPS (Sigma-Aldrich)); (ii) Th1 differentiation, 20 µg/ml Poly I:C (Sigma-Aldrich); (iii) Th2 differentiation, 10 µg/ml PGE2 and 100 ng/ml *E. coli* LPS (Sigma-Aldrich). After 2 days of incubation, DC were washed and incubated with heterologous naïve CD45RA+/CD4+ T cells (naïve CD4+ T cell isolation kit; Miltenyi) ($50x10^3$ T cells/ $5x10^3$ DC). In parallel, DCs were analyzed for maturation markers (CD83 and CD86) by flowcytometry. After day 4, 10 IU/ml rIL-2 was added and every 2/3 days the T cell medium was refreshed with medium containing rIL-2 (10 U/ml). To determine the cytokine production of the Th cells, at day 13 quiescent T cells were restimulated with 30 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) for 6 h. After 1 h, 10 µg/ml Brefeldin A (Sigma-Aldrich) was added to the T cells. Single cell production of IL-4 and interferon-γ (IFN-γ) was determined by intracellular flowcytometric analysis. Cells were fixed in 1x cytofix/cytoperm solution (Beckton Dickson, San Jose, CA) permeabilized with 0.5% saponin (Sigma-Aldrich) and stained with anti-human IFN-γ-FITC and anti-human IL-4-PE (Beckton Dickson).

Results

***S. mansoni* worm glycolipids induce maturation of DC and Th1 response**

To examine the capacity of glycosphingolipids from adult worms (worm glycolipids, WGL) to induce maturation of human DC, immature human monocyte derived DC were stimulated with different amounts of worm glycolipids. Worm glycolipids induce upregulation of the maturation markers CD80, CD86 and HLA-DR in a dose dependent manner (Fig. 1, data shown for CD86 only), indicating that the glycolipids have the capacity to induce maturation of the DC. DCs primed with *S. mansoni* worm glycolipids were incubated with heterologous naïve T cells as described previously [23,34] to asses the capacity of primed DCs to induce a

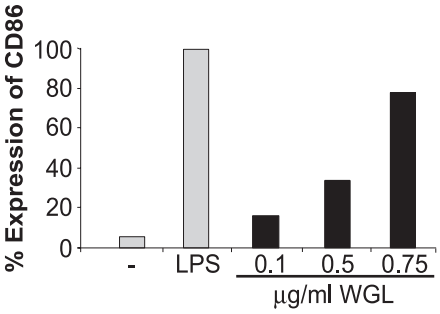

Figure 1: Glycolipids of adult worms induce upregulation of CD86 on human dendritic cells. Human immature dendritic cells (iDCs) were incubated with different concentrations of worm glycolipids (WGL). After 24h, the expression of CD86 on the DCs was determined by FACS analysis, and shown as a percentage of the expression of CD86 induced by 10 ng/ml LPS (100 %). A representative experiment is shown, out of at least three experiments, using DCs derived from monocytes isolated from different blood donors.

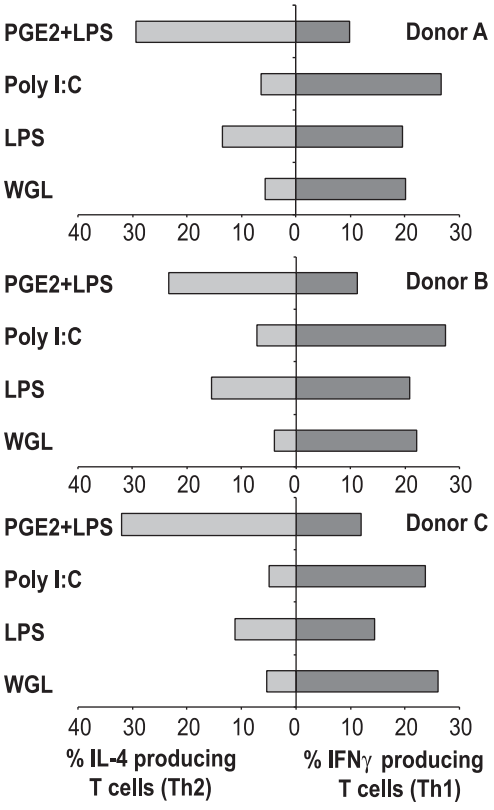

Figure 2: Glycolipids of adult worms skew naive T cells towards a Th1 phenotype. Human immature dendritic cells (iDCs) were incubated with worm glycolipids (WGL) (0.75µg/ml), Poly I:C (20µg/ml) , LPS+PGE2 (100ng/ml and 10µg/ml) and LPS (100ng/ml) for 48 h, washed and co-cultured with heterologous naive $CD45RA^+/CD4^+$ T cells. Naïve T cells were re-stimulated with PMA and ionomycin and the IL-4 and IFN-γ production was measured intracellularly on single cell basis by flow cytometry. Three out of five donors are shown.

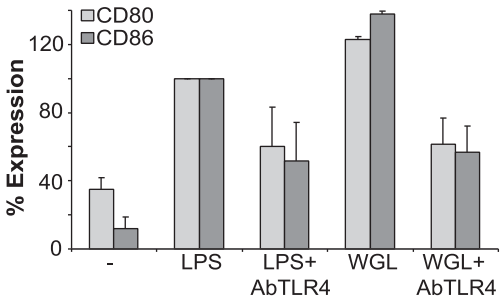

Figure 3: Worm glycolipid-induced dendritic cell maturation is TLR4 dependent. Human immature dendritic cells (iDCs) were incubated with 0.75 μg/ml of worm glycolipids (WGL) in the presence or absence of a TLR4 blocking antibody (10μg/ml). After 24 h, the expression of CD80 and CD86 on the DCs was determined by FACS analysis and shown as a percentage of the expression of CD80 or CD86 induced by 10 ng/ml LPS (100 %). The shown experiment is an average of 2 experiments. An isotype control antibody did not show inhibition of CD80 or CD86 expression.

Th1 or Th2 response. Simultaneously, several controls were included to assess the polarizing capacity of the DCs that were derived from 3 different human donors. LPS induced a mixed Th1/Th2 response in this assay, as deduced from the capacity of the T cells to produce both IFN-γ and IL-4 (Figure 2), whereas Poly I:C induced a strong Th1 profile and Th2 skewing was induced by PGE2 combined with *E. coli* LPS. DCs primed with worm glycolipids consistently induced of naïve T cells to differentiate into IFN-γ producing T cells (Fig. 2). These findings support a role for S. mansoni worm glycolipids in the potential skewing towards a Th1 immune response.

Interaction of *S. mansoni* worm glycolipids with dendritic cell receptors

To identify the putative candidate receptors on iDCs that are involved in the worm glycolipid-induced maturation and Th1 skewing, we explored the possibility that the worm glycolipids would activate TLRs on DC. Human monocyte derived DC were stimulated with 0.75μg/ml worm glycolipids or LPS in the presence or absence of a blocking antibody against TLR4. Both worm glycolipids and LPS induce upregulation of CD80 and CD86 on the DC surface. However, in the presence of a TLR4 blocking antibody the DC maturation was clearly reduced as measured by the reduced expression of CD80 and CD86, indicating that the induction of maturation is dependent on TLR4 (Fig 3). To further investigate the roles of TLR4 and possibly TLR2 signaling, HEK 293 cells expressing either TLR4 or TLR2 were stimulated for 24 h with different concentrations of worm glycolipids, or deglycosylated glycolipids (worm ceramides). As readout for TLR activation, the production of IL-8 by the cell lines was measured. Surprisingly, worm glycolipids or ceramides (in concentration of 10 – 100 μg/ml) were not capable to induce the production of IL-8 from the HEK TLR cell lines, in contrast to the known TLR2 or TLR4 agonists (Pam3CysK4 and LPS, respectively) (data not shown). These data indicate that the worm glycolipids do not directly activate TLR2 or TLR4 in this system.

To evaluate the involvement of other receptors in the initial interaction of DC with worm glycolipids we studied the interaction of C-type lectins. To investigate whether the presence of glycan antigens on the glycolipids are involved in the maturation process, worm glycolipids were treated with HF to remove fucose moieties from the glycolipids, or with endoglycoceraminidase to remove the complete glycan from the ceramide fraction. Defucosylation or total deglycosylation of the worm GL abolished their capacity to induce DC maturation. The results suggest that the glycan structures and in particular the fucosylated antigens are pivotal for induction of DC maturation by schistosome worm glycolipids (Fig. 4A), which may suggest the involvement of a fucose dependent C-type lectin (CLR). To explore whether the fucose-recognizing DC lectin DC-SIGN is involved we performed a cell adhesion assay using a DC-SIGN blocking antibody. The data shown in Fig. 4B demonstrates that DCs strongly bind worm glycolipids and that this binding could be blocked by a DC-SIGN specific blocking antibody, indicating that recognition of schistosome worm glycolipids by DCs is mediated by the C-type lectin DC-SIGN.

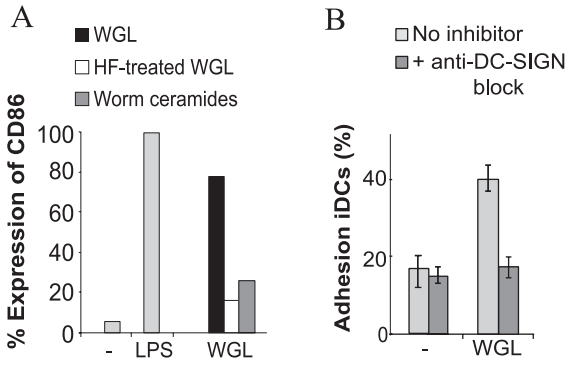

Figure 4: Maturation of adult worm glycolipids is dependent on fucose moieties (A) and worm glycolipids bind dendritic cells via DC-SIGN (B).

A.) Human immature dendritic cells (iDCs) were incubated with worm glycolipids (WGL), HF treated (defucosylated) WGL or the ceramide fraction of the WGL, all at a concentration of 0.75 µg/ml. After 24h, the expression of CD86 on the DCs was determined by FACS analysis, shown as a percentage of the expression of CD86 induced by 10 ng/ml LPS (100 %). A representative experiment is shown, out of at least three experiments, using DCs derived from monocytes isolated from different blood donors. B.) WGL interact with immature DC via DC-SIGN. WGL (10ng/well) were coated in ELISA plate and a solid-phase adhesion assay was performed. iDCs labeled with Calceine AM were added in the presence or absence of a blocking antibody for DC-SIGN, the adherent cells were lysed, and the released fluorescent label measured by spectrofluorimetry. The results are expressed as the mean percentage of adhering cells (100%= total amount of cells added per well). Immature DCs strongly bound to the WGL, whereas no significant binding of the DCs was detected in the presence of the anti-DC-SIGN blocking mAb AZN-D1 (20 µg/ml). The data in B are derived from three independent experiments performed in duplicate or triplicate.

S. mansoni worm glycolipids that bind DC-SIGN express Le^X and LDNF antigens

DC-SIGN typically recognizes both fucose- and mannose-containing glycan antigens [35]. To establish whether fucose moieties on the worm glycolipids are essential for DC-SIGN binding, it was tested whether iDCs recognize defucosylated (HF-treated) worm glycolipids. The data in Fig. 5A show that the iDCs did not bind HF treated worm glycolipids, indicating that DC-SIGN recognizes the fucosylated glycan antigens. DC-SIGN has already been reported to recognize Le^X and LDNF antigens on S. mansoni but so far, the glycan moieties present on

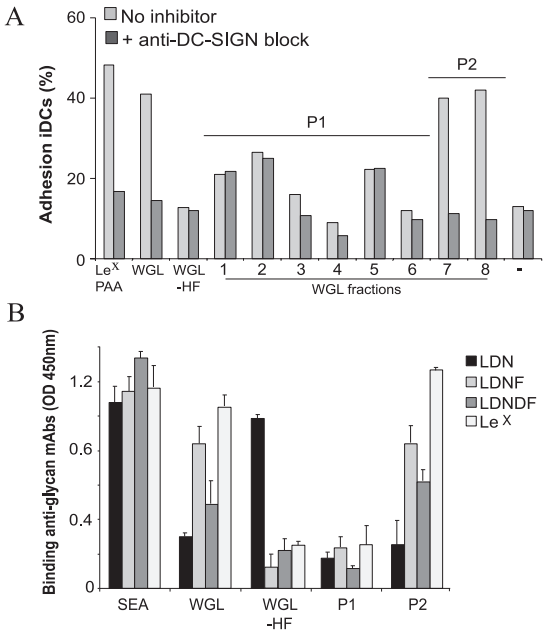

Figure 5: Worm glycolipids that bind dendritic cells via DC-SIGN are enriched by size fractionation and contain high levels of Le^X and LDNF antigen.

Worm glycolipids (WGL) were fractionated into 8 different fractions by silicagel chromatography as described in materials and methods. A.) Immature DCs bind to fractions 7 and 8 of the fractionated WGL via DC-SIGN, as was demonstrated in a plate adhesion assay. WGL (10ng/well) and WGL fractions P1 and P2 (2 ng/well) were coated in ELISA plate and a solid phase adhesion assay with immature DCs, in the presence or absence of a DC-SIGN blocking antibody was performed as described in Fig. 4B. The results are expressed as the mean percentage of adhering cells. Defucosylated (HF treated) WGL were not recognized by iDCs. The fractions that did not bind DC-SIGN were pooled (fraction P1) and the DC-SIGN binding fractions were pooled (fraction P2).B.) WGL and fraction P2 contain high levels of the glycan DC-SIGN ligands Le^X and LDNF, and a moderate level of LDN-DF antigen, as deduced from their reactivity in ELISA with anti-glycan mAbs recognizing LDN, LDNF, LDN-DF and Le^x glycan moieties, respectively. The WGL and HF-treated WGL were coated at 10 ng/well, and the fractions P1 and P2 at 2 ng/well. Schistosome soluble egg antigen (SEA, coated at 10 μg/ml) was used as a positive control.

worm glycolipids have not been reported. To evaluate whether schistosome worm glycolipids contain the known schistosome glycan antigens Le^x, LDNF, LDN and LDNDF, we tested whether antibodies recognizing these glycans bind to the isolated worm glycolipids. These results suggest that the worm glycolipids contain high levels of Le^x and LDNF antigens, which are known DC-SIGN ligands, whereas lower levels of LDN and LDNDF antigens are present (Fig. 5B). The anti-glycan antibodies that require fucose for recognition showed a strongly decreased binding to the defucosylated (HF-treated) glycolipids, whereas increased binding of an anti-LDN antigen was observed indicating that the glycan backbone was not affected by HF-treatment.

Worm glycolipid-induced DC maturation requires DC-SIGN

To investigate a putative role of DC-SIGN in induction of DC maturation, the GL species that are recognized by DC-SIGN were purified. Worm glycolipids were fractionated using a silica cartridge and tested for their ability to bind DCs via DC-SIGN, in a cell adhesion ELISA. As deduced from the results in Fig. 5A, iDCs most prominently bind the glycolipid fractions 7 and 8 and this binding could be inhibited by a blocking antibody against DC-SIGN, indicating that GL in fraction 7 and 8 contained the DC-SIGN glycan ligands. The fractions 1, 2 and 5 showed a low binding to DCs, which appeared to be DC-SIGN-independent, whereas no binding of DCs could be detected to the remaining fractions. The glycolipid fractions that did not bind DC-SIGN were pooled (P1, fraction 1-6) and the DC-SIGN binding fractions were pooled (P2, fraction 7+8). Analysis of the glycan antigens present in fraction P1 and fraction P2 glycolipids using the anti-glycan antibodies showed that only fraction P2 was recognized by anti-Le^x and anti-LDNF mAbs, suggesting the presence of the corresponding glycan antigens in these glycolipids (Fig. 5B). To investigate whether the DC-SIGN binding pooled fraction P2 is capable of inducing iDC maturation, iDCs were stimulated with the pooled fractions for 24 h. As shown in Fig. 6, the level of upregulation of maturation markers on DC induced by glycolipid fraction P2 is comparable to that of the total worm glycolipids. By contrast, the P1 fraction did not induce maturation (Fig. 6A). Furthermore, the induction of DC maturation by total worm glycolipids and fraction P2 is abrogated almost completely by preincubation of the iDC with a DC-SIGN blocking monoclonal antibody, whereas the anti-DC-SIGN antibody did not affect LPS-induced maturation of the DC (Fig. 6A). To investigate whether the interaction of worm glycolipids with DC-SIGN played a crucial role in the production of cytokines by DC, IL-12 p40, IL-10, IL-1β, IL-6, IL-8 and TNF-α of differentially stimulated DCs was evaluated in the presence and absence of anti-DC-SIGN antibody. Our data show that worm glycolipids induce enhanced secretion of all cytokines tested (Fig. 7). The cytokine production induced by glycolipid fraction P2 is comparable to the total worm glycolipids, whereas the glycolipid fraction P1 does not induce secretion of any of the cytokines tested. When DCs were preincubated with anti-DC-SIGN antibody, the observed cytokine production induced by worm glycolipids or with fraction P2 was abolished, in contrast no affect was observed on the LPS induced cytokine production. Summarizing, these data indicate that worm glycolipids

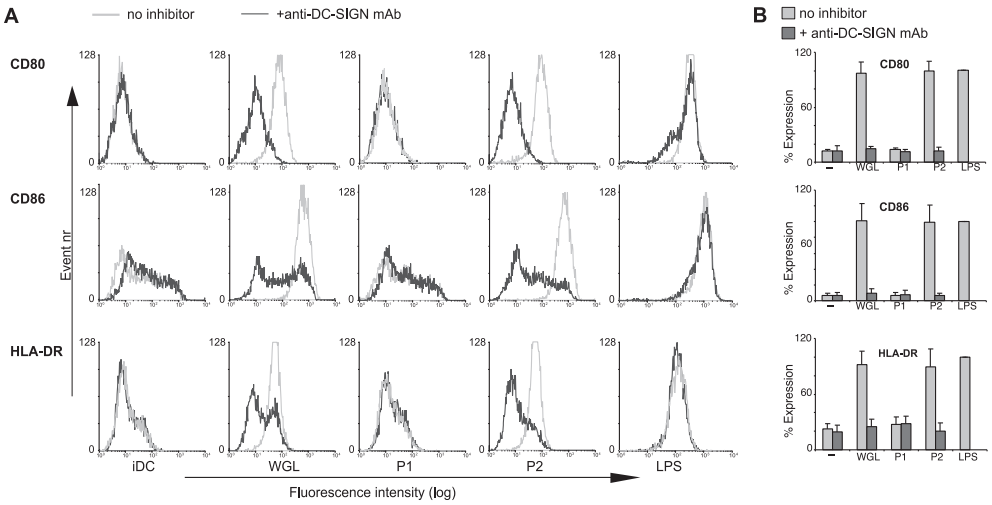

Figure 6: DC maturation induced by worm glycolipids requires interaction with DC-SIGN. Worm glycolipids (WGL) (0.75 µg/ml) induce upregulation of CD80, CD86 and HLA-DR on immature DCs, as was demonstrated by flow cytometry (FACS) analysis after incubation of the iDCs with worm GL, or the pooled worm glycolipid fraction P2 (0.2 µg/ml), for 24 h. No upregulation of any of these maturation markers was detected after incubation of the DCs with these glycolipids in the presence of DC-SIGN blocking antibody AZN-D1 (20 µg/ml). Fraction P1 did not induce upregulation of any of these maturation markers. In panel A, histograms of one representative experiment are shown. In panel B, the mean values of 4 independent experiments are shown, and the values compared to those induced with LPS (100%). Each of the 4 experiments was performed in duplicate with monocyte-derived DCs from a different donor.

have the capacity to induce DC maturation and cytokine production via interaction of fucose-containing glycan moieties, possibly Le^x and/or LDNF, with DC-SIGN.

S. mansoni worm glycolipids interact with DC-SIGN to facilitate TLR4 stimulation

Our findings demonstrate that glycolipid-induced DC maturation is DC-SIGN and TLR4 dependent and that the cytokine response is DC-SIGN dependent. In addition, the worm glycolipids do not trigger TLR4 activation in HEK293 cells that express TLR4. The data therefore led us to explore the possibility that TLR4 activation may require DC-SIGN as a co-receptor. To asses the putative role of DC-SIGN in TLR4 and/or TLR2 activation, DC-SIGN was introduced into HEK 293 cells expressing TLR4 or TLR2 (Fig. 8A). The TLR4 and DC-SIGN expressing HEK293 cells were stimulated with the glycolipid fractions P1 or P2 and the production of IL-8 by the cell lines was measured after 24 h. Fraction P1 was not capable to induce the production of IL-8 in any of the HEK cell lines tested. By contrast, fraction P2 was capable of inducing IL-8 production in HEK293 cells that co-expressed TLR4 and DC-SIGN (Fig. 8B), whereas no IL-8 production was detected by fraction P2 in HEK293 cells co-

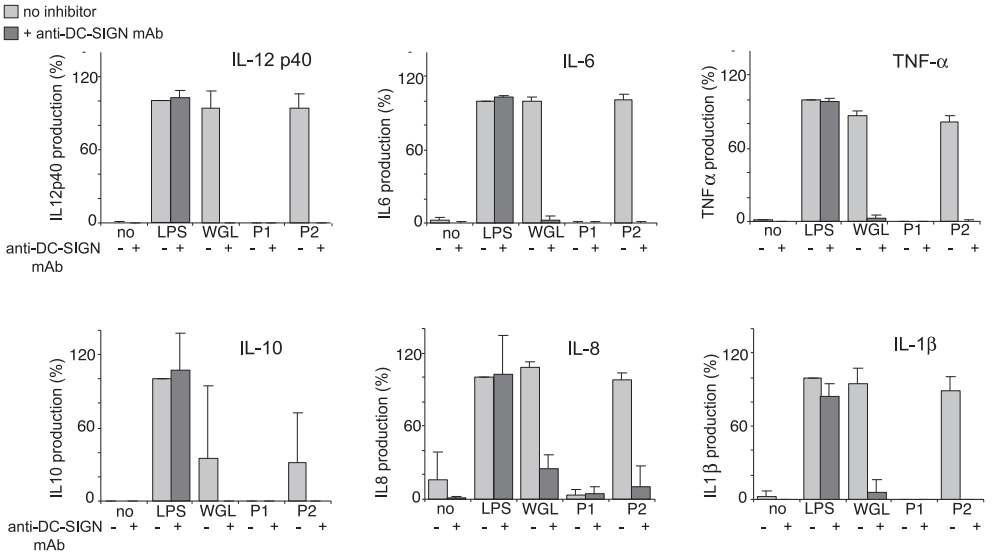

Figure 7: Cytokine production induced by worm glycolipids is dependent on DC-SIGN.
Total worm glycolipids (WGL) and fraction P2 induce the production of IL-12 p40, IL-10, IL-8, IL-6, IL-1β and TNF-α. Supernatants were harvested from cells stimulated for 24h with WGL (0.75 μg/ml), P1 (0.2 μg/ml), P2 (0.2 μg/ml) or LPS (10 ng/ml) in the presence or absence of anti-DC-SIGN mAb (20 μg/ml). The cytokine production was measured by multiplex. The data is the main value of 6 separate experiments.

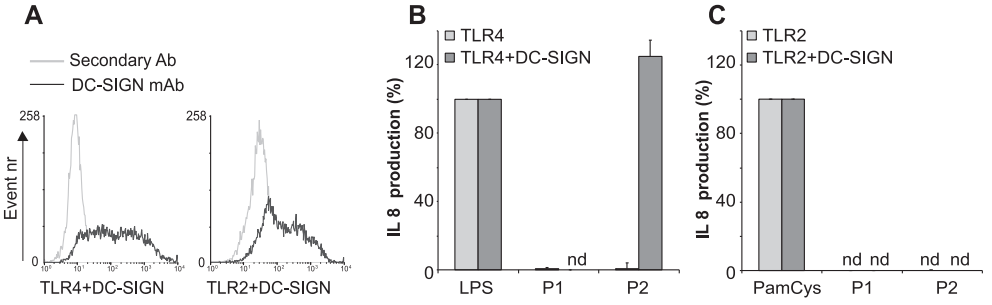

Figure 8: DC-SIGN enables worm glycolipids to activate TLR4.
Worm glycolipid fraction P2, but not fraction P1, induces TLR4 signaling in the presence of DC-SIGN. A.) DC-SIGN was introduced in HEK 293 cells stably transfected with TLR4 or TLR2, and expression of DC-SIGN was determined by FACS analysis using the anti-DC-SIGN mAb AZN-D1. B, C.) HEK293 cells expressing TLR4 (B), or TLR2 (C) with or without co-expression of DC-SIGN were incubated with 0.25 μg/ml of P1 and P2, respectively. TLR activation was determined by measuring IL-8 production after 24 h. The production of IL-8 by 1 ng/ml LPS for TLR4, and 1 ng/ml Pam3Cysk4 for TLR2, were set at 100 %. IL-8 production varied between 200 and 100 pg/ml for LPS-induction in the different experiments and between 1000 and 300 pg/ml for Pam3Cysk4. n.d. : production of IL-8 was below detection level.

expressing TLR2 and DC-SIGN (Fig. 8C), or HEK293 cells expressing TLR2 or TLR4 only (Fig. 8 B,C) or DC-SIGN only (not shown). These data indicate that fucosylated worm glycolipids require DC-SIGN to activate TLR4 in HEK 293 cells.

Discussion

During *S. mansoni* infection, adult worms can survive for many years in the host where they manage to evade the host immune system, despite strong host immune responses to the pathogens. This indicates that a balance is established between host activation and immune suppression. To increase the understanding of the role of helminth glycans in the modulation of host immune responses, the capacity of glycoconjugates derived from different developmental stages of schistosomes to modulate the function of DCs was explored. Here we show that *S. mansoni* worm glycolipids are able to induce maturation of human monocyte derived DC. DCs primed with these glycolipids are able to induce the differentiation of naïve T cells into Th1 cells. Furthermore, our data indicate that the schistosome glycolipids activate immature DCs by a novel mechanism, which involves a DC-SIGN-dependent activation of TLR4, illustrating that DC-SIGN functions as a co-receptor for TLR4 activation.

DCs are the main guardians of the immune system and form a crucial link between the innate and the adaptive immune system. Depending on the recognized antigen, DCs are able to induce naïve T cells to differentiate into different Th subsets. Most pathogens including *S. mansoni*, express different pathogen associated molecular patterns and trigger multiple pathogen-recognition receptors on a single cell. Thus, in many cases the resulting immune response is dependent on the way TLRs and C-type lectins are triggered, and interconnection through their signaling cascades. The current concept is that C-type lectins, by binding pathogen-derived glycans, can deliver signals that influence TLR induced signals, either positively or negatively. Several examples illustrate the cross-talk between TLR and C-type lectin receptors. For example, Chieppa et al. showed that the production of pro-inflammatory cytokines by LPS stimulation of DCs was suppressed by targeting of the MR implying cross talk between the MR and TLR4 [36]. The C-type lectin Dectin 1 on the contrary can act together with TLR2 to enhance the production of TNFα and facilitates Th1 skewing [37]. The C-type lectin DC-SIGN has often been associated with inhibition of TLR-mediated cellular responses against pathogens. For example, mycobacterial component ManLam is recognized by DC-SIGN, inhibits LPS induced upregulation of maturation markers and secretion of IL-12, whereas enhancing IL-10 production [38]. Similarly, *Helicobacter pylori* skews T cell responses towards a Th2 type response via interaction of DC-SIGN with Le^X antigens on *H. pylori* LPS [39]. In addition, it has been reported that ligation of DC-SIGN induces phosphorylation of Erk and Akt, but not p38MAPK, and promotes a transient calcium influx [40,41]. Gringhuis et al. reported that DC-SIGN can signal via Raf-1, which in turn can lead to acetylation of the p65 NF-κB subunit and increased IL-10 production [42]. By contrast, Salp15, a molecule derived from ticks binds DC-SIGN thereby inducing Raf-1/MEK signaling and inhibition of the

production of proinflammatory cytokines in DCs [43]. It is currently unclear how triggering of DC-SIGN leads to differential signaling. Possibly, binding of different glycan ligands which can induce conformational changes of the carbohydrate-binding domain [21] may affect signaling, however the putative contribution of (unknown) co-receptors may not be excluded when using pathogen derived glycoconjugates. In summary, most data and the signaling pathways discovered so far support the concept that triggering of DC-SIGN induces polarization of the immune response towards a Th2 and a regulatory phenotype.

By contrast, our data indicate that schistosome worm glycolipids require DC-SIGN to induce DC maturation and a pro-inflammatory response. The involvement of DC-SIGN in Th1 skewing induced by LPS from the *lgtB* mutant of *Neisseria meningitides*, has been reported previously, however the mechanism was not explored [44]. The strong upregulation of maturation markers, accompanied by the production of many pro-inflammatory cytokines that we observed using the schistosome glycolipids resembled LPS-induced maturation. It should be noted though that the production of IL-10 varied strongly between different donors but was in general lower than the LPS-induced IL-10 production, which is in line with a more pronounced skewing towards a Th1 phenotype by the glycolipids compared to LPS. These data prompted us to investigate the involvement of TLR4. The proposed mechanism by which the worm glycolipids induce DC maturation via a DC-SIGN dependent activation of TLR4, has not been previously reported and is not yet completely understood. For presentation of LPS to TLR4, several accessory molecules are involved [45]. It may be possible that for the schistosome glycolipids, DC-SIGN is needed to catch the glycolipids to present them to TLR4. This mechanism would require physical association of DC-SIGN with TLR4. Both DC-SIGN and TLR4 have been reported to reside within lipid rafts [40,46], which may allow their physical association, but this possibility needs to be further explored. Interestingly, it has been shown that SIGNR1, a mouse homologue of DC-SIGN, recognizes the glycan-core of LPS of several gram-negative bacteria and enhances pro-inflammatory cytokine production, possibly through an observed physical association between SIGNR1 and TLR4 on the plasma membrane [47]. Whereas the schistosome worm glycolipids require DC-SIGN as a co-receptor to activate TLR4, the mechanisms may show similarities in the observed cooperation between TLR4 and a member of the DC-SIGN family to allow or enhance TLR4-induced pro-inflammatory responses.

The capacity of the worm glycolipids to induce DC maturation via a novel pathway raises a strong interest in the structure of these glycolipids. To date, no structural data are reported for the glycan composition of glycolipids derived from adult schistosomes, in contrast to those of schistosomal cercariae and eggs that are well characterized [32,33]. The observation that the worm glycolipids are well recognized by anti-Le^X and anti-LDNF antibodies suggests that the corresponding structures are prominent on the worm glycolipids and may act as DC-SIGN ligands [22,35], however this needs to be established. Less clear is how the worm glycolipids activate TLR4. Our data indicate that glycolipids isolated from schistosomal cercariae do not induce DC maturation (unpublished), despite strong binding to DC-SIGN via Le^X and pseudo-

Le^Y antigens [21]. This suggests that possibly the ceramide portion is important for interaction with TLR4. Thus far, little is known about these ceramides. Differences in the ceramide moieties of mono- and dihexoside ceramide species have been demonstrated between adult worms, cercariae and eggs [48], but these studies do not include the ceramides of the higher molecular weight glycolipids carrying the DC-SIGN-binding fucosylated glycan moieties.

Thus far, the glycolipids of schistosome worms are the first schistosome components shown to activate TLR4, and induce Th1 skewing, in human dendritic cells. The exact molecular basis of the underlying mechanism needs to be further explored. Recognition of LPS by TLR4 is complex and requires several accessory molecules, including LPS-binding protein, CD14 and MD-2 [49]. Using HEK293 cells expressing TLR4, we clearly showed that worm glycolipids could only activate TLR4 when DC-SIGN was introduced as a co-receptor in these HEK cells, in contrast to LPS, which could directly activate TLR4. These data show an essential role for DC-SIGN even in an artificially transfected cell system like HEK TLR4-DC-SIGN. However, the involvement of other factors in DC maturation apart form TLR4 and DC-SIGN cannot be excluded. Interestingly, in the mouse system glycoconjungates containing Lacto-N-fucopentaose III (LNFPIII, a milk sugar carrying a Le^x moiety) induce Th2 skewing via activation of TLR4 [14]. However, this should involve another mechanism of TLR4 activation since no murine Le^X–binding DC-SIGN homologs are known on murine dendritic cells.

In general, helminth infections are associated with a Th2-type of response. However, upon infection schistosomes migrate to the portal vascular system, where male and female worms mate. During this early phase of infection, a Th1 response to the worms is generated. Only after egg-laying this Th1 response shifts towards a dominant Th2 response induced by released soluble egg antigens (SEA) [3]. The potential of adult worm glycolipids to induce DC maturation and the production of a wide array of pro-inflammatory cytokines suggests that in infections such worm glycolipids may contribute to the Th1 response which is characteristic early in schistosome infection.

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CHAPTER 6

General discussion and summary





The group of parasitic worms (helminths) consists of highly diverse organisms belonging to evolutionary very distantly related phyla [1]. In this thesis studies with two of such helminths, the nematode *Haemonchus contortus*, a gastrointestinal nematode of sheep and goats, and the trematode *Schistosoma mansoni*, a human pathogen are described. Despite their evolutionary distance, infection by helminths typically is characterized by host T helper 2 (Th2) and T regulatory responses. Th2 responses are characterized by CD4 T cells, which produce interleukin (IL)-4, IL-5, IL-13 that induce B cells to switch to the production of IgE. In addition, eosinophils, basophils and mast cells contribute to the Th2 response [2]. This Th2 response is not solely dependent on pathological properties of the helminth because free-living helminths are also able to induce a Th2 response [3]. However, helminths are also masters in disguise being able to live in the host for a long time without any major clinical symptoms, thus apparently staying unnoticed by the immune system. Interestingly, the latter effect is even so pronounced that it is extended to bystander antigens, and many data indicate that helminth infections have a beneficial effect on inflammatory immune diseases [4-7]. The first reports unraveling the possible mechanism behind this 'immune suppression' were the counteraction of a Th2 response against pathogens inducing a Th1 response, thereby suppressing the infection. However, the last few years it is becoming apparent that the counteraction of the Th1 response by a Th2 response is not the full story because even Th2 characterized diseases seem to be suppressed by helminth infections indicating towards a role for regulatory T cells (Treg) and their ability to broadly suppress the immune response [7, 8].

Although the characteristic response against helminths is a Th2 response, some helminths also have Th1 inducing stages. The trematode *S. mansoni* primarily induces a Th1 response when the cercariae enter the host and upon egg-laying of the adult worm the Th1 response switches to a Th2 response. The newborn micofiliarial stage of *Brugia malayi* induces a Th1 response in mice although adult worms induce a Th2 response. There are indications that the genetic background of the host plays a pivotal role in the Th1/Th2 balance as indicated in different strains of mice infected with *Trichuris muris* [9].

The location of the parasite infiltration is also pivotal for the triggering of the hosts immune response against the helminth. The immunological activation of gastrointestinal helminths are different from those induced by tissue dwelling helminths although they are both mainly characterized by a Th2 response. In gastrointestinal helminths, like *H. contortus*, the Th2 response is important for expulsion of the helminth [10-12] and their tissue invasive properties are less extensive. In tissue dwelling helminths, like the trematodes *S. mansoni* [13], the Th2 response has a dual role. It protects the host against tissue damage caused by the migrating helminth stages and it protects the helminth from being expelled.

Immune modulation by gastrointestinal nematodes

The characteristic immune response against nematodes/gastrointestinal nematodes is predominantly a Th2 response orchestrated by cytokines including IL-4, IL-5, IL-9, IL-13, IL-21, IL-25 and IL-33, which leads to a cascade of effector mechanisms like IgE producing B cells,

mast cells, eosinophils, basophils and increased permeability, smooth muscle contractility and mucus production in the gut [14, 15]. These are all mechanisms associated with gastrointestinal nematode expulsion, which are favorable for the host but not for the survival of the parasite.

Most nematodes developed mechanisms to circumvent this expulsion. Modulation of the Th2 response is the major mechanism of gastrointestinal helminths to undermine their expulsion, and nematodes developed different mechanisms to counteract this host defence. For example, *H. polygyrus* has developed a mechanism to suppress Th2 cytokines like IL-5 that are involved in the degranulation of mast cells and the induction of Treg [16, 17] cells. *T. muris* on the other hand rapidly downregulates the early Th2 responses in favour of a Th1 response, which is unable to induce the right effectors for the expulsion of the helminth. This latter strategy also holds some danger but if the Th1 is kept under control by for instant the regulatory cytokine IL-10 this is favourable for the helminth [18]. It has also been demonstrated that helminths can shed molecules that mimic self-components of the host [19,20]. *T. muris* for instance expresses molecules that share epitopes with interferon-gamma (IFN-γ), which can bind to the IFN-γ-receptor [21] and induce a Th1 type response. Next to the shedding of components, the helminths can also modulate antigen presentation. The Th2 response is dependent on the expression of antigen in major histocompatibility complex (MHC)-class II molecules. This antigen presentation is dependent on the processing of the antigens by cysteine proteases [22]. Some helminths like *N. brasiliensis* [23] and *H. contortus* [24, 25] have the ability to produce cysteine protease inhibitors or cystatins that inhibit antigen processing. Thus, gastrointestinal nematodes exploit a variety of mechanisms to prevent expulsion and they induce pathology via mall nourishment or blood loss. The long co-evolution between nematodes and their hosts is an indication that parasites are masters in disguise and are able to sustain themselves in a hostile environment.

Treatment of nematode infections

Currently, helminth infections are combated using a variety of natural and synthetic antihelminthics. An urgent problem is that more and more helminth strains become resistant to these drugs. In addition, most of these drugs also do not protect against re-infection with the parasite. Therefore a more long-lasting approach to prevent or fight helminth infections has to be developed. Development of a vaccine would help to solve these two problems. In the past few decades, tremendous research effort focussed on protein based vaccines. In the case of *H. contortus*, there have been some successful trials with native H11 protein, a glycoprotein complex derived from gut membrane cells of adult worms. Unfortunately the immunologic properties of H11 were lost when a recombinant protein was used [26-28]. The difficulties in finding a protein antigen for effective vaccination against helminths and the observation that the major humoral response in helminth infection is directed to glycan antigens [29, 30] contributes to the increasing interest in the role of glycan antigens in helminth infections. In 1978, Nash T.E. et al. already found both IgG and IgM antibodies against gut polysaccharides in patients infected with *S. mansoni* [31, 32] and many reports describing anti-glycan responses in helminth infections have followed [29, 30, 33]. Anti-glycan antibodies have been associated with several cases of host protection

against infection, which indicates the potential of these molecules as a target for vaccine development [34, 35]. Antibodies have been found against fucosylated GlcNAc-GalNAc capped with tyvelose, which are present in the larval stage of *Trichinella spiralis* [35]. These anti-tyvelose antibodies induce expulsion of the larvae and by this way protection [36]. Lambs immunized with *H. contortus* ES glycoproteins, which contains fucosylated LacdiNAc (GalNAcβ1-4-(Fucα1-3) GlcNAc, LDNF) antigen, were protected after challenge with L3 infective larvae and showed a high antibody response to the LDNF antigen that significantly correlated with protection [34]. These data suggest that it could be feasible to use a glycan-based vaccine to prevent disease from gastrointestinal nematodes. An advantage of a glycan based vaccine would be the possibility to develop vaccine components that can be used for multiple helminth infections, since related, but also unrelated nematodes share similar glycan antigens [37].

Helminth glycan antigens as vaccine components

Glycoconjugate vaccines, in which surface carbohydrates from pathogens are covalently attached to an appropriate carrier molecule, have already proven to be an adequate means of inducing a protective immune response in a wide range of microbial diseases. The need for a glycoconjugate vaccine instead of a solely glycan based vaccine is because polysaccharides generally induce an immune response by the cross-linking of Ig receptors on B cells [38-40], without inducing a T cell dependent response. Without this T cell response, there is no induction of immunological memory, avidity maturation and isotype switching of Igs and the produced antibodies are largely IgM [41, 42]. This response is also less capable of inducing the activation of complement. Nowadays, there are some vaccines based on repeating polysaccharides for bacterial infections like the vaccine against *Haemophilus influenzae* type b [43] and meningococcal group A, C, Y and W135 (ACWY Vax®), but the major disadvantage of these vaccines is that they do not induce immunization in infants and they do not induce memory in grownups making it necessary to re-vaccinate frequently.

The improvement of glycan-induced immunogenicity of a glycan structure when it is connected to a carrier protein was first described by Avery and Goebel in 1931 [44]. These vaccines induce immunity in infants and also immunological memory. The immune response elicited against glycoconjungates involves the internalization of the glycoconjugate, and degradation of the carrier protein by proteolytic enzymes in an antigen presenting cell (APC). Appropriate peptides are then displayed by the APC on a MHC II complex making it accessible for the recognition by T cells. T cells then can provide the appropriate signal needed for the class switch in B cells and the induction of immunological memory [45]. The use of glycoconjungates instead of polysaccharides also has the advantage that smaller glycan structures can be used because this process is not dependent on the cross-linking of Ig- receptors on B cells [46-48]. An increasing number of these glycoconjugate based vaccines is being developed and some of them, like *Cryptococus neoformans* [49-51], Group B *streptococcus* [52-54], *Neisseria menigitidis* Group A [55, 56] *Haemophilus influenzae* (ActHib®),... are already used in the clinic or in clinical trials.

One of the key elements of producing glycan-based vaccines for helminths is the characterization of immunogenic glycan structures. *H. contortus* is known for expressing the antigenic structures LDN (LacdiNAc, GalNAcβ1-4GlcNAc) and LDNF (GalNAcβ1-4(Fuc α1-3)GlcNAc) [34, 57, 58]. In **Chapter 2,** *H. contortus* is reported to contain significant levels of Galα1-3-GalNAc antigens on glycoproteins, which are immunogenic in sheep. Vaccination of lambs with ES, which contains this epitope, resulted in the production of anti-Galα1-3-GalNAc Abs as deduced from analysis of serum Abs by glycan micro array, which correlated with protection similarly as found for the anti-LDNF antibodies [34]. Another element important for the production of a glycan based vaccine is the production of the glycan in vitro and the coupling of it to a carrier protein. To enable the evaluation of helminth glycan antigens as vaccine components, we have developed an easy method to synthesize LDN(F) antigens using a chemo-enzymatic procedure **(Chapter 3)**. To generate an acceptor structure, we coupled a fluorescent hydrophobic aglycon spacer, 2,6-diaminopyridine (DAP), to N,N'-diacetylchitobiose. The glycan part was elongated using recombinant *C. elegans* β1,4-N-acetylgalactosaminyltransferase to LDN, and subsequently with human α1,3-fucosyltransferase VI (FucT-VI) to convert the LDN glycan structure to LDNF. The synthesized LDNF-DAP was coupled to carrier protein, via activation of the DAP-moiety by diethyl squarate. Future studies should reveal whether immunization with neoglycoproteins such as described in **Chapter 3** will lead to high levels of anti-glycan Abs and whether these Abs would contribute to protection to infection.

Next to putative application in vaccination, neoglycoconjugates carrying defined glycan antigens could also be used as a diagnostic tool to detect helminth infection. This may be advantageous to current diagnostical methods that often rely on visually counting the parasite eggs in the stool, or by echography, which can only be done when the infection is in a more developed stage (increased pathological consequences). The anti-glycan antibody response measurement could make the detection easier and earlier.

Tissue dwelling parasites

The characteristic Th2 response seen in gastrointestinal nematode infection is also seen in tissue-dwelling nematodes like filarial nematodes [59] and schistosome [13] infection. The expulsion/ protecting mechanisms related to this Th2 response as observed in gastrointestinal nematodes is not so clear-cut for tissue dwelling nematodes [60, 61]. The Th2 response in tissue dwelling parasites is associated with formation of fibrotic tissue as seen around the eggs of *S. mansoni* [62] and in *onchocerra* [63] infection and is frequently involved in pathology in the chronic phase of these infections as seen for *S. mansoni*.

By contrast, in tissue dwelling helminths the Th1 responses are implied in the resistance and killing of larval stages and the suppression of migration of the helminths [61, 64]. However, an overactive Th1 response is harmful to the host. This is apparent in humans suffering from acute schistosomiasis also known as Katayama fever, which in some patients happens before egg laying (Figure 1). The immune cells of these patients produce high levels of tumor necrosis

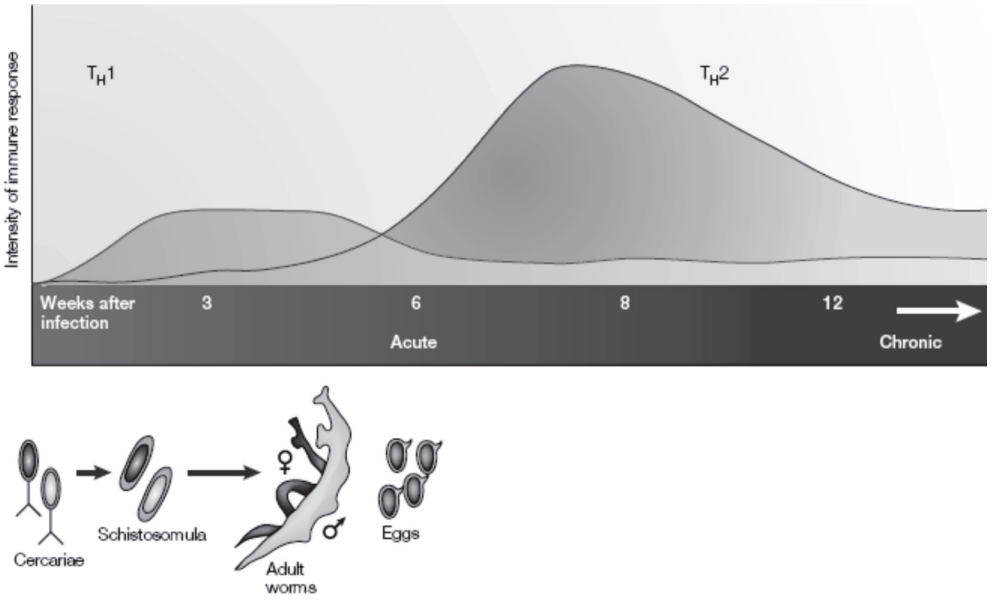

Figure 1. Development of the immune response during *S. mansoni* infection.
During the first 3-5 weeks after the infection, the immune response against *S. mansoni* is predominantly Th1. As the parasite matures and egg production starts, the Th1 response decreases and the Th2 response increases. The Th2 response is mainly induced by egg antigens and can become chronic of nature if the disease is not treated. Adapted from Pearce et al. [64].

factor (TNF), IL-1 and IL-6, which are cytokines connected to a Th1 response. The severity of the disease drops if the immune response switches to a Th2 response induced by egg-antigens. In mice models the inability to induce a Th2 response is fatal. This lethality is suggested to be connected to the inability of tissue remodeling after egg migration, indicated by the increased levels of lipopolysaccharides and nitric oxide levels in the bloodstream of these mice [65, 66]. The immune-switch from Th1 to a Th2/T regulatory response in schistosoma infections is thus beneficial for the survival of both the parasite and the host.

S. mansoni glycans in immune responses

The last few decades, it is becoming increasingly clear that there is a strong humoral response against schistosome glycan epitopes on larvae, adult and eggs [31, 32, 67]. In humans and mice infected with *S. mansoni*, elevated levels of glycan antibodies were reported. In human and mice antibodies against the LDN, LDNF, Le^X (Galβ1-4-(Fucα1-3)GlcNAc, $Lewis^X$) and LDN-DF (GalNAcβ1-4(Fucα(1-2)Fucα(1-3)) GlcNAc)[68-73] were found. The serum levels against LDN-DF were higher in comparison to the other structures possibly indicating that this structure is more immunogenic than the structures like LDN and LDNF. The elevated serum levels against Le^X is slightly surprising because this epitope is also present in the host but could maybe be

explained by the difference in carrier molecule, or unusual presentation of the Le^X antigens such as in poly- Le^X structures. Next to the glycan specific antibody production, it is also becoming increasingly clear that glycans play a role in the induction of a Th2 response during helminth infections [74, 75].

Because there are more and more indications that glycans play a role in *S. mansoni* infection [74-77], it is important to understand the interactions of these glycans with the immune system. Receptors that could play an important role in these interactions are glycan binding proteins (lectins), like galectins and C-type lectins. These two lectin families are expressed by immune cells and may represent putative links between the helminth and the host immune system that contributes to induction of the helminth -specific immune responses.

The involvement of galectin 3 in S. mansoni infection - Galectin 3 (Gal-3) is a member of the galectin family, known to bind β-galactosides. From the 14 mammalian galectins reported until now, Gal-3 is one of the best studied members and it has been implied in the immune response during helminths infections with both positive and negative outcomes for the host [78]. In earlier studies, it has been shown that an elevated amount of Gal-3 was present in liver granulomas surrounding the eggs of *S. mansoni* [79]. The authors also showed that Gal-3 could bind LDN glycans and soluble egg antigens (SEA) of *S. mansoni*. In a follow-up study, the Gal-3 gene was knocked out in mice, and the mice were infected with *S. mansoni.* Interestingly, Gal-$3^{-/-}$ mice had a more pronounced Th1 response in comparison to their wild type counterparts. We showed that the Gal-$3^{-/-}$ displayed increased anti IgG2b levels against SEA, and lower IgG1 levels in comparison to wild type animals. Investigation of the dendritic cells (DC) of these Gal-$3^{-/-}$ mice indicated that these cells may not be the direct cause of the observed effects, but the Gal-$3^{-/-}$ DC showed an elevated ability to induce T cell activation and proliferation, compared to wild type DC [80]. It is unclear how the lack of Gal-3 influences dendritic cell function in these studies. In addition, it is not known how Gal-3 expression and secretion is regulated. To increase the insight in the putative role of Gal-3 on DC function, we investigated whether human DCs can bind Gal-3, and which factors regulate Gal-3 expression and secretion in human DCs (**Chapter 4**). Whereas our findings could not shed much light on the above observations, the results provided novel insights in Gal-3 biology in human monocyte-derived DCs. We showed that Gal-3 expression and secretion is regulated by IL-4 and GM-CSF. Our data showed that IL-4 is a strong inducer of Gal-3 mRNA expression, whereas addition of GM-CSF inhibited the IL-4-induced upregulation of Gal-3 mRNA expression. The total Gal-3 protein level was similar in the different stimulated monocytes, suggesting a posttranscriptional regulation of Gal-3 protein production. Remarkably, only the GM-CSF stimulated monocytes showed significant amounts of secreted Gal-3. In immature DCs, however, hardly any release of Gal-3 was found, indicating that IL-4 has a suppressive effect on GM-CSF induced Gal-3 secretion. Summarizing, the data showed that Gal-3 expression and secretion in immature DCs is very restricted and highly regulated, and the cells hardly can bind extracellular Gal-3. Possibly, the intracellular Gal-3 expression in immature DCs and the reduced extracellular binding may

be protective against Gal-3 induced phosphatidylserine (PS) exposure or apoptosis, however this needs to be established. We hypothesize that GM-CSF induced Gal-3 secretion, which in its turn may induce the influx of monocytes and macrophages [81] may facilitate a broad influx of inflammatory cells. This process could be involved in liver granuloma formation around *S. mansoni* eggs, where large amounts of Gal-3 have been reported [79].

Induction of Th1 responses in S. mansoni infection. - During the infection with *S. mansoni*, the primary response is Th1 which shifts towards a Th2 response upon egg laying [64]. To increase the insight in the mechanisms by which helminth glycans contribute to shaping the immune response of the host, the role of C-type lectins and Toll-like receptors (TLR) on DCs in their interaction with glycolipids of adult worms was investigated (**Chapter 5**). We showed that *S. mansoni* worm glycolipids induce maturation of DC as deduced from the upregulation of maturation markers and the production of pro-inflammatory cytokines. Co-culture of glycolipid-primed DCs with naïve T cells skewed T cell responses towards a T helper cell type 1 profile. We demonstrated that the maturation induction here was dependent on both TLR4 and DC-SIGN. We also showed that only the DC-SIGN binding fractions, which contain Le^X and LDNF glycan antigens induced maturation. We hypothesize that the worm glycolipids are caught by DC-SIGN, which binds to the glycan moieties of the glycolipids and that upon this binding the lipid part of the glycolipid is able to activate TLR4 (Figure 2). In this model, DC-SIGN thus acts as an essential coreceptor for TLR4 activation, which has not been reported before. This type of CLR-dependent activation of a TLR would require colocalization of both receptors. Colocalization between TLR4 and another C-type lectin has been shown for mSIGNR1, a mouse homolog of DC-SIGN, during stimulation with the LPS of a gram-

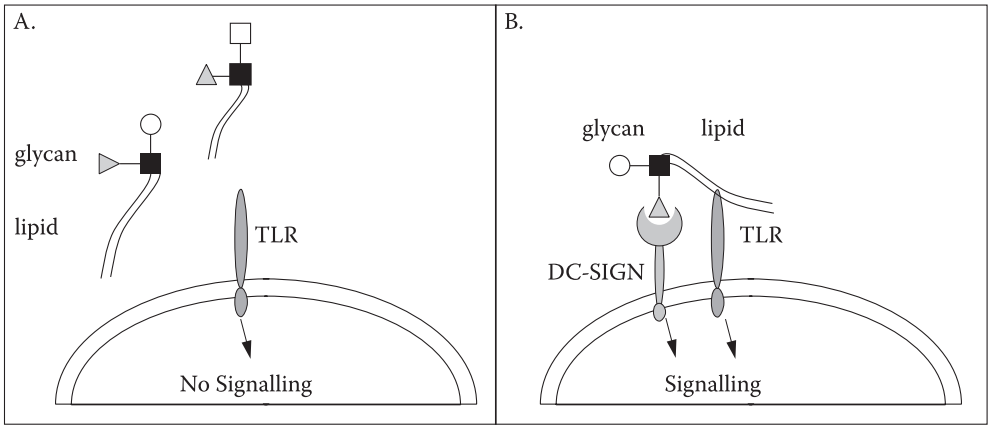

Figure 2. Model DC-SIGN-TLR4 cross-talk.
A.) Glycolipids are not able to induce TLR4 signalling by themselves. B.) We hypothesize that the worm glycolipids are caught by DC-SIGN, which binds to the glycan moieties of the glycolipids and that upon this binding, the lipid part of the glycolipid is able to interact with TLR4.

negative bacterial pathogen, however SIGN-R was not required for TLR4 activation in this case [82]. Although the mechanism proposed here should be investigated in more depth, our data provide evidence that the DC-SIGN-dependent TLR4 activation is a novel way of CLR-TLR cross-talk.

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Nederlandse samenvatting





Helminth glycanen en hun interactie met het immuunsysteem

Infecties met parasitaire helminthen (wormen) tasten de gezondheid aan van mens en dier, en vormen derhalve zowel een medisch als economisch probleem. Parasitaire infecties kunnen ertoe leiden dat de gastheer te weinig voedingstoffen binnen krijgt en/of te veel bloed verliest. Daarnaast kunnen er andere medische complicaties optreden, zoals lever cirrose bij *Schistosoma mansoni* infectie. In het ergste geval kunnen parasitaire infecties leiden tot de dood van de gastheer. De meeste parasitaire infecties kunnen tegenwoordig bestreden worden met antiparasitaire middelen. Deze hebben echter heel vaak als nadeel dat zij geen bescherming vormen tegen herinfectie en het komt ook steeds frequenter voor dat de parasiet een resistentie ontwikkelt tegen deze geneesmiddelen. Daarom wordt het alsmaar belangrijker om nieuwe technieken te ontwikkelen om deze infecties te bestrijden, bijvoorbeeld door vaccinatie. Hierbij is het van belang dat er onderzocht wordt hoe de parasiet communiceert met het immuunsysteem van de gastheer. De afgelopen decennia is de meeste aandacht uitgegaan naar de interactie van helminth eiwitten met het immuunsysteem van de gastheer, maar in de afgelopen jaren wordt het steeds duidelijker dat ook glycanen (suikermoleculen) bij deze interactie een belangrijke rol spelen.

Cellen die een cruciale rol spelen bij de herkenning van worm antigenen (lichaamsvreemde componenten) zijn antigeen presenterende cellen (APC). Deze cellen zijn in staat hun omgeving te scannen en antigenen te herkennen. Na herkenning kunnen ze het antigeen opnemen, afbreken en vervolgens in stukjes presenteren op hun celoppervlakte zodat de effector cellen van het lichaam zoals T-cellen en B-cellen een signaal krijgen om het antigeen op te ruimen. De dendritische cel (DC) is een heel belangrijke APC. DCs zijn in staat om hun omgeving te herkennen via verschillende receptoren zoals Toll-like receptoren (TLRs) en lectines. TLRs zijn oppervlakte moleculen die geconserveerde structuren kunnen herkennen in pathogenen zoals lipopolysaccharide (LPS), wat een onderdeel is van de celwand van bacteriën. Herkenning van deze structuren induceert een signaal in de cel waardoor de DC wordt geactiveerd om de effector cellen van het lichaam aan te trekken en aan te sturen. Lectines, die zowel uitgescheiden kunnen worden als op het oppervlakte van DCs tot expressie kunnen worden gebracht, zijn daarentegen gespecialiseerd in de herkenning van glycanen. Er bestaan veel verschillende lectine receptoren. In deze thesis wordt onderzoek gepresenteerd betreffende twee subsets van lectines: C-type lectines en galectines. C-type lectines zijn vooral bekend omdat ze in staat zijn om componenten uit de omgeving te internaliseren. Er zijn echter sommige C-type lectines, zoals DC-SIGN, die ook in staat zijn te signaleren en op die manier de afweerreactie te beïnvloeden. Galectines zijn oplosbare lectines die extra- en intracellulair kunnen voorkomen. Een van de meest bestudeerde galectines is galectine-3 (Gal-3). De invloed van Gal-3 op het immuunsysteem is zeer veelzijdig. Het is bijvoorbeeld betrokken bij de celdood van immuuncellen, de secretie van cytokinen en het aantrekken van sommige effector cellen. Daarnaast is er van Gal-3 ook bekend dat het Galβ1-4GlcNAc (LDN) herkent, een structuur die veel gevonden wordt in helminthen. Ook hebben ze in voorgaande

onderzoeken aangetoond dat Gal-3 voorkomt in de laesies die ontstaan rond *Schistosoma mansoni* eitjes in de lever van de gastheer. Dit zijn beiden aanwijzingen dat Gal-3 een rol speelt in de afweerreactie tegen helminthen

In deze thesis worden de moleculaire interacties belicht die helminth glycanen hebben met het immuunsysteem van de gastheer. Het onderzoek werd uitgevoerd met twee verschillende wormen, namelijk de gastro-intestinale nematode *Haemonchus contortus* (**Hoofdstuk 2**) en de trematode *Schistosoma mansoni* (**Hoofdstuk 5**). In **Hoofdstuk 2** wordt aangetoond dat het serum van schapen, die beschermd waren tegen infectie na vaccinatie met *H. contortus* ES proteïnen (uitgescheiden eiwitten), immunoglobuline G (IgG) antilichamen bevat die de glycaan antigenen Galα1-3-GalNAc-R en GalNAcβ1-4(Fucα1-3)GlcNAc- (LDNF) herkennen. Met behulp van specifieke antilichamen hebben we ook aangetoond dat *H. contortus* glycanen naast Galα1-3-GalNAc- structuren ook Galα1-3-Gal structuren bevatten. De afweerreactie die ontstaat tegen deze glycanen is een indicatie dat deze structuren als zijnde lichaamsvreemd worden herkend. Deze structuren zouden dus eventueel in de toekomst gebruikt kunnen worden in de context van een vaccin om de afweer tegen *H. contortus* infectie te verhogen. **Hoofdstuk 3** omschrijft een methode om de helminth glycanen GalNAcβ1-4GlcNAc (LDN) en α3-gefucosyleerd LDN (LDNF) te synthetiseren. Hiermee hebben we de mogelijkheid gecreëerd om bepaalde glycaan structuren op grotere schaal te synthetiseren. Dit maakt het mogelijk om de specifieke interactie van deze glycanen met het immuunsysteem te bestuderen waardoor duidelijker wordt hoe dergelijke glycanen de afweerreactie van hun gastheer beinvloeden. Daarnaast zouden deze structuren eventueel ook gebruikt kunnen worden als component van een vaccin tegen helminthen.

Uit eerder werk is bekend dat het gastheer lectine Gal-3 een belangrijke rol speelt bij de interactie van helminth glycanen met het immuunsysteem. Om meer inzicht te krijgen in de rol van Gal-3 in helminth infecties hebben we in **Hoofdstuk 4** gekeken naar het expressie profiel van Gal-3 in DCs. We hebben deze DC subset *in vitro* verkregen na de stimulatie van monocyten met de cytokinen GM-CSF en IL-4. In dit hoofdstuk is vooral belicht welke invloeden deze twee cytokinen hebben op de Gal-3 expressie en secretie. Hieruit blijkt dat de verhoogde mRNA expressie van Gal-3, die geïnduceerd wordt door IL-4, wordt onderdrukt door GM-CSF en dat GM-CSF de secretie van Gal-3 induceert, hetgeen geremd wordt door IL-4. Dit geeft aan dat Gal-3 expressie en secretie zeer afhankelijk is van het samenspel van deze cytokinen. Daarnaast zien we ook dat de oppervlakte expressie van Gal-3 op monocyten bijna volledig verdwijnt na stimulatie met IL-4 en/of GM-CSF. Dit zou erop kunnen wijzen dat de DCs die gevormd worden minder gevoeling zijn voor de extracellulaire effecten van Gal-3, zoals celdood, hetgeen belangrijk is voor de functie van een DC.

In **Hoofdstuk 5** wordt bewijs geleverd dat glycolipiden geïsoleerd uit *S. mansoni* volwassen wormen de maturatie van DC en een T helper 1 respons induceren. De resultaten laten zien dat de worm glycolipiden deze respons faciliteren via de activatie van TLR4 en zo een ontstekingsreactie induceren. Interessant is dat de worm glycolipiden niet rechtstreeks TLR4 kunnen activeren, maar alleen wanneer ze gelijktijdig binden aan het C-type lectine DC-SIGN.

In de glycolipiden fractie, die in staat is deze activatie te induceren, zijn glycolipiden aanwezig die Galβ1-4(Fucα1-3)GlcNAc- (Le^X) en LDNF glycaan antigenen bevatten. Uit deze resultaten kunnen we afleiden dat het glyaan gedeelte van de worm glycolipiden een interactie aangaat met DC-SIGN en dat de binding aan DC-SIGN cruciaal is voor de activatie van de DC via TLR4.

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Curriculum Vitae





Curriculum Vitae

Caroline Margretha Wilma van Stijn geboren op 4 oktober 1981 in Maaseik België. Na het behalen van haar diploma Wetenschappen-Wiskunde op het Koninklijk Atheneum te Maaseik in 1999, begon zij met de studie Biomedische wetenschappen aan het Limburgs Universitair Centrum (tegenwoordig Universiteit Hasselt) te Diepenbeek. Na de kandidatuurjaren, zette zij haar studie verder aan de transnationale Universiteit Limburg (tUL; samenwerking tussen Diepenbeek en Maastricht), waar zij in 2004 haar diploma 'licentiaat in de Biomedische wetenschappen/ Doctorandus in de gezondheidswetenschappen' behaalde. Tijdens deze studie deed ze stageonderzoek naar "T-cel homeostase bij autoimmuunziekte". Dit onderzoek werd uitgevoerd in het Biomedisch Onderzoeksinstituut aan het Limburgs Universitair Centrum en de transnationale Universiteit Limburg te Diepenbeek (België) onder de supervisie van Prof. dr. P. Stinissen. In 2005 begon zij als AIO op de afdeling Moleculaire celbiologie en immunologie aan het VU medisch centrum, onder de begeleiding van Prof. dr. Y. van Kooyk en dr. I.M. van Die. Het promotie onderzoek werd verricht naar de interactie van helminth glycanen met het immuunsysteem, zoals beschreven staat in dit proefschrift.





List of publications

Boris Tefsen, Caroline M. W. van Stijn, Marloes van den Broek, Hakan Kalay, Jaco C. Knol, Connie R. Jimenez, Irma van Die. Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF. Carbohydr. Res. (2009).

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